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EVALUATING THE EVOLUTIONARY AND GENETIC RELATIONSHIPS OF THE
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BY
KYLIE BUCALO

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By

Kylie Bucalo

Committee Chair:

Dr. Kevin Burgess

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Dr. Jennifer Cruse-Stander

Alex Reynolds

EVALUATING THE EVOLUTIONARY AND GENETIC RELATIONSHIPS OF THE DNA BARCODING IN ANDEAN ORCHIDS OF NORTHWESTERN ECUADOR

By

Kylie Bucalo

Committee Chair:

Dr. Kevin Burgess

Committee Members:

Alvaro Pérez

Dr. Jennifer Cruse-Sanders

Alex Reynolds

Index words: Ecuador, Orchid, DNA barcode, *Pleurothallis*, *Epidendrum*, *Stelia*, *Lepanthes*, *Oncidium*.

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THESIS ABSTRACT

DNA barcoding is a molecular based technique used to separate and identify individual species. Here we establish a DNA Barcode library for the orchid flora of an Andean cloud forest in Northwestern Ecuador. The library contains 135 *matK* and 136 *rbcL* DNA Barcodes representing over 33 Orchidaceae genera. Sequence analysis shows percent species resolution was higher for *matK* (98.8%) than *rbcL* (70.24%), with a large portion of the unresolved species for the *rbcL* loci coming from taxonomically complex genera in the subtribe Pleurothallidinae. Neighbor Joining (NJ) trees revealed that the orchid flora of Siempre Verde is divided taxonomically into two large monophyletic clades at the sub family level; Orchidoideae and Epidendroideae. Sequences within Orchidoideae presented with high bootstrap support across all NJ trees (*matK*, *rbcL* and *matK+rbcL*), indicating species within the clade are well resolved. Resolution for sequences within sub family Epidendroideae varied depending on taxonomic clade and loci used. Overall the *matK* NJ tree outperformed the *rbcL* NJ tree by delivering monophyletic clades at the subfamily, tribe, and subtribe level with higher bootstrap values, separating a higher number of congeners, particularly those in taxonomically complex genera such as *Pleurothallis*, *Stelis*, and *Lepanthes*. Estimates of evolutionary divergence showed a very low level of intraspecific variation in DNA Barcodes of target cryptic species *Oncidium heteranthum*, acknowledging that floral traits in *Oncidium* are often highly plastic, and not indicative of species lines.

Index words: Ecuador, Orchid, DNA barcode, Pleurothallis, Epidendrum, Stelis, Lepanthes, *Oncidium*.

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INTRODUCTION

DNA barcoding is a molecular tool that involves sequencing standardized loci to obtain a short section of DNA that can be used for species identification (Herbert et al., 2003a). The loci sequenced are different across plants, animals, fungi, protists and algae (Kress and Erickson, 2012). In animals, the mitochondrial gene cytochrome c oxidase I (COI), has been widely adopted as the universal barcode, however, the region fails to work in plants, primarily because of the low nucleotide substitution rate in the mitochondrial genome (Hebert et al., 2003b, Hebert et al., 2003a, Kress et al., 2005, Fazekas et al., 2008, Hollingsworth et al., 2009). Unlike animals, plant DNA barcoding usually requires a multi locus approach involving loci from coding (*matK*, *rbcL*, *rpoB* and *rpoC*,) and non-coding regions (*trnH-psbA*) of the plastid or nuclear genomes (ITS) (Kress et al., 2005, Chase et al., 2007, Fazekas et al., 2008, Fazekas et al., 2009).

As a phylogenetic tool DNA Barcoding can be used to delimitate species, clean up the cladistics of genera and subgenera within a family, identify new species and examine the evolutionary relationships between species (Erickson and Driskell, 2012). As a taxonomy tool, barcoding is useful for species identification, particularly when material is scarce, degraded, or ephemeral in nature (Kress and Erickson, 2012). The ability of DNA barcoding to handle non-traditional samples and morphologically complex groups, coupled with the dwindling number of trained taxonomists makes it a critical additive tool for species identification, delimitation and classification, in large and complex plant families such as the Orchidaceae.

Untangling the complex relationships present in the Orchidaceae family has traditionally been a taxonomy issue, conventionally involving differentiation via morphological traits of the column, and the pollen type, as seen in Dressler's classification schemes (Chase et al., 2003, Dressler, 1993). However issues arise with morphology based taxonomy in orchids, because

floral traits in some genera have a high level of intraspecific variation, and are prone to selective pressures from pollinators (Kim et al., 2014, Cameron et al., 1999). Molecular systematics has been able to aid traditional taxonomic efforts by using genetic analysis to reclassify the Orchidaceae within all levels of the family's phylogeny. In high order lineages, molecular studies have used full length markers to reclassify sub families, in lower order phylogenetic groupings, whole gene markers and barcodes are used to sort out complex relationships between sub tribes, associations in and between genera, and to also investigate taxonomic organization of subgenera. (Cameron et al., 1999, Whitten et al., 2000, Pridgeon et al., 2001, Koehler et al., 2002, Chase et al., 2003, Cameron et al., 2004, Freudenstein et al., 2004, Cameron et al., 2006, Sheade et al., 2012, Whitten et al., 2014). Within the lowest phylogenetic orders of the Orchidaceae family, DNA barcoding has been used to assess genetic variation in congeneric species (Yao et al., 2009, Xiang et al., 2011, Singh et al., 2012) identify new species (Bogarín et al., 2007 and Pessoa et al., 2012) and detect illegal orchid trade (Subedi et al., 2013). Lastly, and most important to this study, DNA Barcoding can be used to catalogue species richness in areas of high orchid biodiversity (Lahaye et al., 2008).

With over 4000 orchid species, Ecuador has the highest orchid diversity in the world (Mites, 2008). A combination of the cooling effects of the Humboldt Current, the warming effects of El Niño, and the topographical effects of the Andean uplift have caused the proliferation of orchid species in Ecuador. (Meisel et al., 2014, Mites, 2008). Orchids can be found in many regions of the country, however one of the largest concentrations can be found at mid level elevations in Andean cloud forests. Present within these environments are high levels of available water, immense elevational gradients, and topographical effects of high ridges and deep valleys, which all give way to the creation of specialist microclimates where orchids thrive

(Meisel et al., 2014, Reynolds, 2004). With over 40% of the country's orchid species being classified as endemic it is clear to see why research, documentation, and identification of the flora is key, particularly as many species in cloud forest ecosystems have become threatened by deforestation. As a taxonomic and phylogenetic tool DNA barcoding can identify and catalogue the species present, provide a molecular library for further research, and examine genetic and evolutionary aspects of the unique flora present.

The goals of this study were to develop a DNA Barcode library of the orchid flora of Siempre Verde, Ecuador, to assess the efficacy of DNA barcodes to demarcate Andean orchid species, to evaluate evolutionary and genetic relationships among complex genera present at the reserve, and lastly to investigate possible cryptic speciation in an *Oncidium* complex.

Taxon sampling

To develop an orchid DNA Barcoding library for Siempre Verde collections were made throughout an elevational gradient from approximately 6,500m to 11,500m and at multiple flowering times to ensure a wide taxonomic dispersion. Collection priority was given to deep sampling amongst genera in the sub tribe Pleurothallidinae, as was repeated sampling across the preserve of *Oncidium bicoloratum* to investigate cryptic speciation. Identification to genus was made in the field upon collection, and later to species if possible. Herbarium vouchers were processed at night after collection, in wooden plant presses, and left to dry for 48 hours in a field made dryer. Specimens were checked every 8 hours to prevent molding and press rotation was

METHODS

Study site

Siempre Verde is a privately owned and protected preserve in the Imbabura province of Northwest Ecuador (See Figure 1). Located on the western foothills of the Cotacachi volcano in the Intag river valley, the 825-acre Preserve is dedicated to plant and animal conservation, scientific research, student education, and service. Scientific research is made possible by the Robert and Connie Braddy Cloud forest Research Station. The preserve contains high elevational cloud forest and regenerating secondary forest between 7500 and 11,000 feet, at the highest point on the property the vegetation can be described as "ceja andina" or "elfin forest" where stunted twisted trees, moss and some epiphytic orchids are present. Temperatures at the research station range from approximately 6 to 24 degrees Celsius year round, and precipitation data taken from Los Cedros, another Intag Valley preserve, dictates average yearly precipitation of 2884.3 mm. Collections were taken along or near cleared hiking trails at Siempre Verde between March 2014 and June 2015, except for samples taken off herbarium specimens.

Taxon sampling

To develop an orchid DNA Barcoding library for Siempre Verde collections were made throughout an elevational gradient from approximately 6,500ft to 11,000ft and at multiple flowering times to ensure a wide taxonomic dispersion. Collection priority was given to deep sampling amongst genera in the sub tribe Pleurothallidinae, as was repeated sampling across the preserve of *Oncidium heteranthum* to investigate cryptic speciation. Identification to genus was made in the field upon collection, and later to species if possible. Herbarium vouchers were processed at night after collection, in wooden plant presses, and left to dry for 48 hours in a field made dryer. Specimens were checked every 8 hours to prevent molding and press rotation was

key to minimize uneven specimen drying. Orchids with pseudobulbs present were dissected before pressing, by making a vertical cut down the length of the organ and carving out any fleshy material whilst being careful to maintain overall bulb morphology. All voucher specimens used in and created by this study, are accessioned in the herbarium at Pontificia Universidad Catolica del Ecuador in Quito, Ecuador. Material for molecular analysis was collected in the field, and stored in silica gel until processing, with the exception of 18 samples, which were lifted off alcohol preserved herbarium vouchers.

DNA Extraction, amplification and sequencing

Molecular leaf tissue taken from either dried silica samples, or herbarium specimens was placed inside tube racks in a DNA Barcoding Sampling Kit from the Canadian Centre of DNA Barcoding (CCDB). Tube racks were then sent to CCDB for DNA extraction, PCR, and bidirectional Sanger sequencing for *rbcL*+*matK* DNA barcoding regions. Extraction, replication and sequencing were performed at CCDB according to standard CCDB protocols. Specific *matK* and *rbcL* primer sets are given in table 1.

Species resolution of DNA barcode library

To assess the efficacy of the DNA barcode library to demarcate Andean orchids a custom BLAST service was created in Geneious® (version 9.0.5)(Burgess et al., 2011). Three local sequence databases, one for each loci (*matK* and *rbcL*) and one for the concatenated sequences (*matK* + *rbcL*) was created. Each sequence was then individually queried against the appropriate database in an all-to-all BLAST. Only sequences belonging to samples identified to species were used in this analysis. When a sequence was found to be unique (query only matched to itself, or a sequence from the same identified species) the sequence was scored as 100% resolved

for that particular gene region. Percentage species resolution for a given gene region was then calculated as the percent of species that had unique sequences.

Phylogenetic analysis of complex genera

To evaluate the evolutionary and genetic relationships among complex genera in the Orchidaceae, nucleotide data was downloaded from BOLD and imported into Geneious as a FASTA alignment. Sequences were then individually removed from the FASTA alignment by using the extract feature. Complete coding sequences for the outgroup species *Curculigo capitulata* for both *matK* (1563 bp) and *rbcL* (1400bp) were downloaded from Genbank. For concatenation, sequence data was manually checked to select only samples that had both loci successfully sequenced. Barcodes were then individually selected and matched to each other for head to tail concatenation. Each paired loci sequence was individually concatenated using the concatenate sequence feature in Geneious, with *matK* leading and *rbcL* following in the head to tail formation. Iterative multiple sequence alignments were completed using Multiple Sequence Alignment by Log expectation (MUSCLE) in Geneious using the software's default settings (Edgar, 2004). Alignments were then checked and manually edited in Geneious, including deletion of selected sequences from the analysis and also trimming sequence lengths of outgroups to fit average barcode sequence length. MUSCLE alignments were then re-run with the modified outgroup sequences, before being exporting to Molecular Evolutionary Genetics Analysis version 7 (MEGA) for tree building . Overall mean genetic distance was calculated for both loci alignments using the distance menu in MEGA, as was average pairwise distance of the outgroups to comment on fitness of outgroup and overall sequence divergence.

Neighbor joining trees were created using the Maximum Composite Likelihood evolution model. The MCL method estimates all distances for a given set of aligned sequences

simultaneously (instead of independently), and does so under the Tamura Nei (1993) substitution model (Tamura, 2004), (Hall, 2011) (Hall, 2013). Gaps and missing data were treated by pairwise deletion, and the tree phylogeny was tested with 2000 bootstrap (BS) replications. All trees were edited using tree drawing tools in MEGA, including labeling tree sections and nodes, flipping subtrees, coloring branches, and collapsing and expanding subtrees. This was done to enhance trees readability and allow for easier comparisons between loci.

Analysis of cryptic speciation

To investigate the *Oncidium* complex to reveal cryptic speciation, estimates of evolutionary divergence between sequences of *Oncidium heteranthum* were calculated. Alignments for both *matK* and *rbcL* were built in Geneious for samples denoted as *Oncidium heteranthum* and the number of differences was calculated by using a pairwise distance matrix in MEGA.

RESULTS

Taxon sampling

A total of 179 samples, representing 33 genera of the Orchidaceae were collected at the Siempre Verde preserve (see Table 2). Of the total collected, 174 samples were identified to genus, and 115 were further identified to species by the authors (Figure 2). Five samples labeled, as Orchidaceae unknown were not identified past family, due to damaged partial specimens that had little or no floral morphology. Targeted sampling of taxonomically complex genera in the subfamily Epidendroideae, resulted in 30% of the genera found in the subtribe Pleurothallidinae having one or more species represented in this collection (see Figure 3). Repetitive sampling of *Oncidium heteranthum* across the preserve resulted in twenty individuals collected for the study.

Sequence recoverability

To discuss DNA Barcoding in the Orchidaceae sequence recoverability was summarized from the information provided by CCDB through the Barcode of Life Database (BOLD), with particular focus given to trace file quality of the failed sequences and the occurrence of stop codons. In total the study added 271 Orchidaceae DNA barcodes to the Barcode of Life database, representing an overall sequence recovery rate of 76% (see figure 4). Recovery was higher for *rbcL* (76%) than *matK* (75%), however the difference equated to only one additional barcode sequence for the *rbcL* loci. From the recovered sequences 64% were from samples that were identified to species, of which 69% are novel to the BOLD database. They are the first molecular record for the species at the time of this publication. The remaining 31% of species identified barcodes, had 6 or less molecular records present in BOLD.

Recovery rates resulted in a loss of 44 *matK* sequences and 43 *rbcL* sequences from the study. For *matK* 22% of the un-sequenced samples did not generate either forward or reverse

trace files, indicating Sanger sequencing was not completed at CCDB for these samples (See Figure 5). Of the other un-sequenced samples for *matK*, 61% gave at least one trace file (forward, or reverse) that was rated as failed, and the last 17% gave both trace files rated as low, medium or high, but did not contain any trace files marked as failed (See Figure 5). For *rbcL* 21% of the un-sequenced samples did not generate either forward or reverse trace files, indicating that these samples never underwent Sanger sequencing at CCDB (See Figure 5). Of the other un-sequenced samples for *rbcL* 67% gave at least one trace file (forward, or reverse) that was rated as failed, and the last 12% had both trace files rated as low, medium or high, but did not contain any trace files as failed. Stop codons were present in 32 (24%) *matK* sequences, and were not present in any sample sequences for the *rbcL* loci.

Species resolution

Results from the all-to-all BLAST to the DNA barcode library showed percent species resolution was higher for *matK* (98.8%) than it was for *rbcL* (70.24%), however highest resolution came from the multi locus concatenation, where full resolution was achieved for every species (100%). Species resolution by genera for *matK* shows 100% of the unresolved species came from the genera *Pleurothallis*, however this percentage only constitutes a single failed barcode, *Pleurothallis grandiflora*. The genera as a whole had a species resolution of 91% for this locus. Resolution by genera for *rbcL* shows 48% of the non-resolved species coming from *Pleurothallis*, the genera as a whole failed to resolve well with this plastid marker with only 31% of its species showing full resolution. Other genera which did not resolve well from subtribe Pleurothallidinae were *Ida*, *Odontoglossum*, *Stelis* and *Trichosalpinx* which all had no samples that were fully resolved (See Figure 6).

Phylogenetic analysis

The final number of sequences included in each alignment, excluding outgroup *Curculingo capitulata*, was 132 each for *matK* and *rbcL*, and 115 for the *matK* + *rbcL* concatenation. Four sequences each were deleted from both *matK* and *rbcL* alignments, and 6 from the concatenated alignment because of truncated sequence length. After these spurious sequences were removed, outgroups were trimmed to match average barcode sequence length. For *matK* outgroup sequence positions 1-410 and 1335-1571 were deleted, for *rbcL* 849 bp were deleted from the outgroup sequence from position 553-1402. Genetic distance estimates show the overall mean genetic distance for sequences in the *MatK* alignment (excluding outgroup) is 39.5, and the outgroup has an average pairwise difference of 127.4. For the *rbcL* alignment the overall mean genetic distance for sequences (excluding outgroup) is 8.3, and the outgroup average pairwise difference is 20.5. Data is given in number of base pair differences.

rbcL Neighbor Joining tree

A total of 133 nucleotide sequences equaling 553 positions became the final dataset for the rooted *rbcL* Neighbor Joining (NJ) tree (See Figure 7). The tree placed sampled genera in two distinct monophyletic clades labeled by the authors in Figure 8 as Orchidoideae, and Epidendroideae. Support for the position of these clades was given by 2000 bootstrap replications, resulting in both clades having bootstrap values >50%. The Epidendroideae clade separates into two distinct groups, which are labeled by the authors as Epidendroideae 1, and Epidendroideae 2, this term is used simply to refer to each group and has no taxonomic reference (See Figure 8).

Despite having relatively high inner node support the Epidendroideae 2 clade shows poor outer branching and outer branching order support. Only a portion of outer branches (7) show bootstrap values > 50 (See Figure 9). Poor resolution in this clade leads to unresolved congeners in many areas of the tree including within the genus *Epidendrum*. Particular focus is given to genera from the subtribe Pleurothallidinae where the tree struggles to demarcate between at least 5 different identified species of *Pleurothallis*. This section of the tree has such poor resolution species from 4 different genera *Pleurothallis*, *Lepanthes*, *Stelis* and *Trichosalpinx* do not separate from each other. Branch lengths depicting genetic distance for many sections of this part of the tree (not displayed) show distances of zero. This is congruent with results from the all-to-all BLAST for this locus.

Epidendroideae 1 appears as a clade in its current position in 87% of replicated trees, and has slightly better outer node support than Epidendroideae 2 as depicted by the bootstrap values (See Figure 10). It is successful at separating this clade at the genus level particularly with respect to clustering species of *Odontoglossum*, *Oncidium*, and *Cyrtorchilum* with high bootstrap values ($>50\%$), however resolution beyond this hierarchy is moderate, as seen with the failure to demarcate between *Odontoglossum cirrhosum* and *Odontoglossum hallii*. High support exists for the position and branching order for the small clade containing *Maxillaria*, *Xylobium*, *Ida* and *Telipogon*, which are identified in the *matK* tree as tribe Maxillarieae.

The Orchidoideae clade gave lowest inner node support for clade position, however it gave some of the highest support for branching within a clade, indicating the species within the clade are well resolved (See Figure 11). Unknown Orchidoideae samples were placed on the tree with relatively high support, 63% of trees positioned KB 125 near *Sauroglossum andinum* and 51% of trees positioned KB185 near several *Odontoglossum* samples. Additionally many

samples that were identified to genus only were placed with high support amongst sections of the tree where possible identifications could be made.

Collapsing branches with less than 50% bootstrap support to show the Majority Rule tree results in a large number of polytomies existing in the Epidendroideae 2 clade (See Figure 12). Higher support is present for Epidendroideae 1 at many internal nodes, the largest polytomy present in this clade results from the repeated sampling of *Oncidium heteranthum*, as branching order cannot be determined when sequences are identical. Computing the majority rule tree does not affect the clade represented by genera in the Orchidoideae, as bootstrap support was high in the original tree. The only polytomy present is between the three unidentified *Erythrodes* species.

matK Neighbor Joining tree

A total of 132 nucleotide sequences equaling 924 positions became the final dataset for the rooted *matK* Neighbor Joining tree (See figure 13). Overall this tree outperformed the *rbcL* tree by delivering monophyletic clades at the sub family, tribe and subtribe level with higher bootstrap values (See Figure 14). Support for the monophyletic position of the sub family Orchidoideae was 97 in the *matK* tree, compared with 56 in *rbcL*, similarly values for internal branching within the clade are also much higher in the *matK* tree (See Figure 16). Node support for tribe Sobralieae in *matK* is more than double than that of *rbcL*, and the trend continues with higher bootstrap support in *matK* over *rbcL* for subtribe Laelinae, Pleurothallidinae, and Oncidiinae. The *matK* NJ tree also outperforms *rbcL* by separating a higher number of congeneric species, particularly those in taxonomically complex groups such as genera found in the sub tribe Pleurothallidinae (See Figure 16.) In this section of the tree *matK* is successful in

separating at least 6 different species of *Pleurothallis*, and 3 species of *Lepanthes* with high branch support (>50%).

Unlike the *rbcL* tree the *matK* tree does not show a bifurcating node that easily splits Epidendroideae into two clades. Instead the tree shows species that were present in *rbcL*'s Epidendroideae 1 clade as evolutionary descendants of species present in Epidendroideae 2 (See Figure 17). Replicated samples of *Oncidium heteranthum* show the same topology as the *rbcL* tree with no genetic difference in the majority of the samples. It is interesting to note that for one genus in the Oncidiinae tribe the *matK* tree does not do as well as the *rbcL* tree. *MatK* tree does not resolve *Cyrtorchilus* species well, the species are paraphyletic in this tree, however they are monophyletic in the *rbcL* tree.

Unknown Orchidaceae samples were placed on the tree with higher support, 87% of trees positioned KB 125 near *Sauroglossum andinum* and 67% of trees positioned KB185 near several *Odontoglossum* samples. Additionally many samples that were identified to genus were placed with high support amongst sections of the tree where possible identifications could be made. Collapsing branches with less than 50% bootstrap support to show the Majority Rule tree demonstrates a high level of monophyly for sub family Orchidoideae, tribes Malaxideae, Maxillarieae, and Sobralieae, and sub tribe Laeliinae (See Figure 18). The majority of polytomies found in Oncidiinae are due to repeated sampling of *Oncidium heteranthum*. Subtribe Laeliinae shows weakened bootstrap support (<50%) for separation of 3 *Epidendrum* species. Sections of subtribe Pleurothallidinae remain monophyletic after computing the majority rule tree more so than *rbcL*, however some sections that are dominated by mostly unidentified *Pleurothallis* and *Stelis* do not, here we still see polytomy in the clade.

Concatenated Neighbor Joining tree

A total of 115 nucleotide sequences equaling 1480 positions became the final dataset for the rooted concatenated Neighbor Joining tree (See Figure 19). The concatenated tree shows identical topology to the *matK* tree, with marginally higher support for tribe Maxillarieae and subtribe Laeliinae and slightly lower support for Cranichideae, and Oncidiinae (See figures 20-23).

Cryptic species

Estimates of evolutionary divergence between *rbcL* sequences of *Oncidium heteranthum* shows that there are zero base pair differences between 17 sequences. *MatK* shows only one divergent sequence, sample number KB189 has one base pair different to the other 19 sequences of *Oncidium heteranthum* included in the loci matrix.

DISCUSSION

Recovery of DNA Barcodes in the Orchidaceae

In this study recovery rates show that a quarter of potential sequences were lost when averaging sequence failure across both loci. Some of the losses (*rbcL* 21%, *matK* 22%) were attributed to an inability to extract DNA or replicate during PCR, and no trace files were provided by CCDB to the authors for those sequences. A high proportion of these samples were sourced from ethanol treated herbarium specimens. This is a practice familiar to tropical botanists, who use this technique to protect plant specimens from fungal spores in hot, wet climates during collection (Ballick et al., 1996). Previous research has found that the practice of treating field specimens with preservatives such as ethanol, accelerates the rate of DNA breakdown (Doyle and Dickson, 1987), and that the extent of DNA degradation in dried herbarium specimens appears to be primarily related to the condition of the fresh leaf tissue when dried rather than the year it was collected (Drabkova et. al., 2002, Rogers and Bendich, 1985). Of the 18 samples collected from herbarium vouchers treated with ethanol, 7 failed to give trace file data, and 3 gave traces that failed for both loci where no sequence was built. It is known that the ethanol is an efficient cloud forest specimen preserver, however our study shows it inhibits successful DNA sequence recovery. Instead it is advised that plants are either sampled before immersion in alcohol, using a small piece of leaf tissue placed in silica for storage, or a plant dryer must be utilized to dry the entire specimen. Preliminary trials during the last field trip at Siempre Verde showed how a modified primitive field plant dryer can be set up with minimal supplies, and vouchers inside presses will completely dry out after 48 hours, which prohibits fungal contamination and preserves DNA well.

Although the high throughput CCDB protocol is very efficient at processing a large number of samples, the inability to repeat or fine-tune DNA extraction and replication processes on failed samples severely limits sequence recovery rates. Similarly, the limited capacity for making informed decisions on contiguous sequence building from CCDB quality ranked trace files, further limits researchers abilities to troubleshoot problem sequences, and ultimately decreases the number of final sequences in a study. Most importantly it is unclear to the authors why a portion of the un-sequenced data for both *matK* (17%) and *rbcL* (12%) gave at least one trace file (forward or reverse) that were equal to or greater than the quality of other trace files in which sequences were found to be barcode compliant. A deeper understanding of the CCDB protocols on trace quality, contig building, and decision-making trees for barcode compliancy is needed. It is understood that some of these drawbacks are inherent when molecular processes are not carried out on site.

Stop codons were present in 24% of *matK* sequences, which is common for this gene region in orchids, and has led many researchers to conclude that *matK* may be present as a pseudogene (Kocyan et al., 2008, Kores et al., 2001). Premature stop codon prevalence is so widespread in this plant family that a quick search by Barthet et al. (2015) of Genbank's *matK* pseudogene marked sequences, revealed a staggering 82% belonging to Orchidaceae (Barthet et. al., 2015). However, other researchers argue that the gene is still functional, that sequence characteristics such as a high level of frame shift mutation and non-synonymous substitution is not enough to warrant classification as a pseudogene (Barthet and Hilu, 2007). The evolutionary explanation given from such researchers is that the family has undergone an evolutionary shift for expression of the *matK* gene, and that an alternative initiation codon (aic) can be used for translation when sequences display truncated non-functional proteins. (Barthet et. al., 2015,

Barthet and Hilu, 2007). These studies show that translating the *matK* orchid sequences using the *aic* instead of the consensus monocot codon (*cic*), caused 80% of the taxa previously reported to contain stop codons, to produce a full length reading frame. (Barthet et al., 2015).

Efficacy of matK and rbcL DNA barcodes to demarcate Andean orchids

Results from the all-to-all BLAST analysis, show that the *matK* loci is the more robust plastid marker for species level identification in Northwestern Andean orchids. The marker resolved 98.8% of samples, with its only failed sequence matching to just one other congener. Additionally the overall mean genetic distance for sequences in the *matK* alignment was higher (39.5 nucleotides) than the mean genetic distance for *rbcL* sequences (8.3 nucleotides). The success of the *matK* barcode can be attributed in part to its characterization as a rapidly evolving gene. The *matK* gene region experiences a rate of nucleotide substitution that is three times higher than that of *rbcL*, creating high levels of interspecific variation as is seen in the genetic distances given above (Barthet and Hilu (2007), Barthet et al., 2015, Johnson and Soltis., 1995).

Previous barcoding studies involving many diverse genera of land plants have shown high levels of species resolution for this barcode, however some state that the marker often requires the use of multiple, or specifically designed primers (Fazekas et al., 2008, Layahe et al., 2008, Kress et al., 2009). In the Orchidaceae the marker was tested on a large dataset of Mesoamerican orchid species (>1000), and species monophyly analysis showed correct species identification reached >90% (Lahaye et al., 2008). In the study the plastid marker amplified and aligned well, was able to correctly identify threatened species of *Phragmipedium* and also helped to reveal cryptic species of *Lycaste*. The *matK* marker is also used as a benchmark locus for resolution comparisons when new gene regions or barcodes are being proposed for use in the orchid family (Neubig et al., 2008). Lastly the *matK* gene region has shown a discriminatory

capability within genera that are taxonomically complex, where traditional morphology based taxonomy fails to separate species well. The marker was able to resolve eight of twelve congeneric species within taxonomically complex *Holcoglossum*, showing the highest discriminatory ability among all single gene regions tested. (Xiang et al., 2011). This is consistent with results in this study where sequences in complex genera such as *Pleurothallis*, and *Lepanthes* were resolved well with the *matK* loci.

In comparison, results from the all-to-all BLAST show the *rbcL* loci failed to separate congeneric species for many genera, and also failed to delimitate between species from different genera. In the genus *Pleurothallis*, many sequence queries matched 100% to 5 or more other congeners, and also matched to species identified in genera *Lepanthes* and *Trichosalpinx*.

Phylogenetically conservative *rbcL* is known to show a low level of discriminatory power below family or sub family levels in many plant families, because of its slow synonymous rate of substitution and its functional constraints. (Kress et al., 2009, Hasebe et al., 1994, Burgess et al., 2011, de Vere et al., 2012). The marker is often paired with other more evolved barcodes for optimal performance in species delimitation. This is congruent with the difference in species resolution seen between *rbcL* and the concatenated (*matK* + *rbcL*) sequence in this study. In Orchidaceae the full-length *rbcL* marker is often restricted to higher order phylogenetic analysis, where it has shown a high level of discriminatory power to differentiate at sub family or tribe level (Cameron et al., 1999, Chase et al., 2003, Cameron et al. 2004, Freudenstein et al. 2004, Cameron et al. 2006,). As a barcode the marker has shown it is not variable enough below the genus level often resulting in low interspecific variation when compared with *matK* (Lahaye et al., 2008, Xiang et al., 2011,). This is congruent with the findings in this study.

Understanding genetic relationships between Andean orchids

Outgroups and sub families

The outgroup *Curculigo capitulata* was chosen to root the Neighbor Joining tree based on previous phylogenetic work that identifies the family Hypoxidaceae as a close relative of the Orchidaceae (Rudall et al., 1997, Kocyan et al., 2004). The species has also been included as an outgroup in several prominent Orchidaceae phylogeny papers (Kocyan et al., 2004, Cameron et al., 1999). Looking at overall mean distance of sequences for both alignments, and the average pairwise distances for the outgroups, *Curculigo capitulata* is a suitable outgroup for use in this study as it is more distantly related to the in-group sequences than the in-group sequences are to each other, however not so primitive that homology cannot be detected (Hall, 2011).

The Neighbor Joining trees generated in this study show the orchids of Siempre Verde being placed into one of two large monophyletic clades (sub-groups), labeled Epidendroideae and Orchidoideae based upon the genera within the clades. These two groupings represent two Orchidaceae sub families, and the grouping of SV genera into these two subfamilies is constant across all types of trees and all loci. In all trees there is high bootstrap support for the monophyly of Orchidoideae, and its position nearest to the outgroup is constant among trees, showing that the subfamily is more primitive than Epidendroideae. This is congruent with the results from previous molecular studies that define the Orchidaceae sub families as five primary monophyletic clades Apostasioideae, Cypripedioideae, Vanillioideae, Orchidoideae and Epidendroideae in that evolutionary order (Cameron et al., 1999, Cameron et al., 2004, Freudenstein et al., 2004, Cameron et al., 2006).

In the genera that form Epidendroideae the *rbcL* tree clearly shows a bifurcation, which splits the sub family into two groups, whereas the *matK* and concatenated NJ trees show members of *rbcL*'s Epidendroideae 1 as evolutionary descendants of genera in Epidendroideae 2.

The two groups in the *rbcL* tree do not correlate with “lower” and “higher” Epidendroideae as classified by Cameron et al., 1999, the only genera this collection contains from “lower” Epidendroideae are *Elleanthus* and *Sobralia*. These genera should form a monophyletic clade that positions on the tree before genera from “higher” Epidendroideae (Cameron et al., 1999). This is congruent with the *matK* tree’s placement of tribe Sobralieae, which sits on the tree immediately after the sub family Orchidoideae, with bootstrap support for the monophyly of the clade at 99%. The remaining genera found in the Epidendroideae are in the “Higher Epidendroideae” sub family.

Subtribe Pleurothallidinae and taxonomically complex genera

The subtribe Pleurothallidinae accounts for approximately 15-20% of the species in the Orchidaceae, and has proven to be extremely difficult to describe (Pridgeon et al., 2001). Species count in the subtribe has increased from an estimated 4000 in 1986 to just over 5100 in 2016, with a larger portion of species being held in genera *Stelis*, *Lepanthes*, *Maxillaria* and *Pleurothallis* (Karremans, 2016). Circumscription of the subtribe is particularly challenging because both morphological and anatomical features used to characterize or group species into taxonomic units often occur in clearly unrelated species. More specifically homoplasy in floral traits between loosely related taxa is strongly attributed to selection pressures by pollinators (Karremans, 2016), (Pridgeon et al., 2001). The staggering number of species present in the subtribe, the presence of diminutive inflorescence, and the presence of homologous traits across genera, characterize members of the subtribe Pleurothallidinae as taxonomically complex genera (TCG). Molecular circumscription of Pleurothallidinae did not exist until fairly recently with the first attempt in 2001 by Pridgeon et al., 2001. This work attempted to assess the monophyly of the subtribe and the genera within. Theirs and other more recent phylogenetic papers will be

discussed in the framework of species found at Siempre Verde to discuss tree topology of the *matK* NJ tree, and comment on genetic relationships within the Pleurothallidinae.

The Siempre Verde orchid flora collected for this study contained 82 samples from subtribe Pleurothallidinae, 54 of which were identified to species. The clade presents in the *matK* Neighbor Joining tree above subtribe Laeliinae and below tribe Malaxideae in the sub family Epidendroideae. Bootstrap support for the clade is 87%. This position is congruent with the genetic relationship presented between Pleurothallidinae and Laeliinae in previous research, where they have usually been considered sister groups, with some disagreement with inclusion of particular “bridge” genera (*Dilomilia* and *Neocogniauxia*) that seem to consistently get moved between the two subtribes (Cameron et al., 1999, Karremans 2016, Pridgeon et al., 2001). In this study the *matK* NJ tree shows the subtribe Pleurothallidinae can be split into four clades, marked on Figure 24 as A, B, C and D, and will be discussed below.

Clade A contains only 3 species, with low bootstrap support for the inner most node, and high BS support as you travel toward the tips. The two species of *Dracula* separate well with the *matK* barcode, as does *Dracula* from *Andinia*. In general this clade resolves well, separating the three species with high bootstrap support. If we are to subscribe to the proposed generic affinities in Karreman’s (2016) paper this clade would consist of affinities *Masdevallia* for *Dracula* and *Specklinia* for *Andinia*. Interestingly *Andinia pensilis* is placed as a sister clade to a clade containing *Pleurothallis* and *Stelis* in previous research using ITS data, similar to the relationship of the topology seen here in the *matK* tree between clades A and B (Cameron et al., 1999).

Clade B is perhaps the “messiest” part of the subtree, with many branches giving fairly low support, and large sections of the tree becoming polytomic, when the majority rule tree is computed (see Figure 25). *MatK* is unable to separate any of the identified species in clade B at

>50% BS support indicating very low confidence in the position of these species on the tree. As Clade D resolves well for the genera *Pleurothallis*, pairwise matrices were analyzed for nucleotide variation between *Pleurothallis dunstervillei* in Clade D and *Pleurothallis sclerophylla* from Clade B (See table 3.) There is a difference of 14 nucleotides between congeneric sequences from different clades. This is compared with a difference of 4 between sequences within clade B when *Pleurothallis sclerophylla* is compared with *Stelis piperina*. Clearly *Pleurothallis sclerophylla* is genetically closer to *Stelis piperina* than another congener from Clade D. This infers that *Pleurothallis* is polyphyletic or some species of *Pleurothallis* should be circumscribed into *Stelis*. This is a very contentious taxonomic question, and is not suitable to answer from a NJ tree with such poor support. To fully resolve species in Clade B without further taxonomic research on the samples additional loci need to be tested, as the concatenated *rbcL* + *matK* NJ tree gave no better resolution than *matK* alone. Authors were fairly conservative when assigning species identification to samples both in the Pleurothallidinae and across the collection, so it is surprising that Clade B cannot resolve at least between *Stelis piperina*, *Pleurothallis sclerophylla*, and *Stelis pusilla*, even if both genera are currently grouped into the affinity *Pleurothallis*, and known to be sister clades in some phylogenetic studies (Karremans et al., 2016, Cameron et al., 1999). Additionally the large number of samples placed on the tree at genus level in this clade makes the tree far less informative, as the only samples with branch support >50% are not identified past genus. The lack of species level samples, coupled with poor BS support indicates that this section of the tree should be retested and not relied upon for confident estimates of genetic relationships between *Pleurothallis* and *Stelis*. Lastly the authors would like to mention that after the tree was analyzed and it was clear that Clade B posed many research questions they returned to field notes taken during collection to

recover any preliminary species identifications for samples that were positioned within Clade B and denoted to genus only. Using these preliminary field identifications a clearer picture emerged that Clade B may contain species of *Pleurothallis* that have been placed into the subgenus *Crocodeilanth*. If this is correct, it lends support as to why specific *Pleurothallis* samples may appear in a clade alongside species of *Stelis* as currently the subgeneric *Crocodeilanth* is genetically very similar to *Stelis* and many species that had previously fallen under this grouping have been recircumscribed into *Stelis* (Karremans, 2015). Additionally none of the *Pleurothallis* species that appear in Clade D fall under this subgenera, which further lends support to this idea.

Clades C and D were well resolved using DNA Barcoding. For example, Clade C resolves very well for genera *Lepanthes* and *Trichosalpinx*, showing high phylogeny support particularly for *Lepanthes*. The tree is able demarcate between 3 species of *Lepanthes*, and infer from branch length the identity of “genus only” sample AP6972 as *Lepanthes mucronata*. *Trichosalpinx* is noted to be paraphyletic in many molecular studies, in our study it is resolved inside Clade C, however this study only has 3 samples, and they are present with poor levels of phylogeny support, collapsing to a polytomy in the majority rule tree (Karremans, 2016). Clade D comprises mostly of well separated identified *Pleurothallis* species with high bootstrap support. In this clade we see resolution of 5 different *Pleurothallis* species, and also an erroneous addition of an unidentified species of *Maxillaria*.

Overall the matK barcode separated the complex genera and species of the subtribe Pleurothallidinae well, with the obvious taxonomic obstacles of too many unidentified species in some areas leading to ambiguities in monophyly. The resolution of Clade B is very poor and investigations into the samples within this clade should be made.

Subtribe Oncidiinae

Species boundaries in the subtribe Oncidiinae are known to be historically contentious, because traditional circumscriptions relied on floral morphology and pollination systems, which have been described as labile. (Neubig et al., 2012). Taxonomic circumscription has been particularly difficult in species containing, yellow “oncidiod” flowers such as *Oncidium heteranthum*, because of floral trait variation in color and shape due to malpighiaceae oil mimicry (Neubig et al., 2012). Recent molecular generic circumscriptions recognize 61 clades in the subtribe, including the separation of *Oncidium*, *Odontoglossum*, and *Cyrtorchilum* as monophyletic genera (Neubig et al., 2012). These three genera have often been tangled together in previous morphological based circumscriptions, because of reliance on floral morphology as generic characteristics. Separation of these three closely related genera, however, is not as clear within the tribe Oncidiinae in this studies NJ tree (See Figure 17).

Lower bootstrap values, and a high level of unidentified species make the tree less informative for the genus *Oncidium*. *Cyrtorchilum* fails to resolve well with two samples of *Cyrtorchilum flexuosum* forming its own clade with high BS support (100), however *Cyrtorchilum serratum* is found elsewhere on the tree making the genera paraphyletic. Lastly *Odontoglossum* does present as a monophyletic clade with high bootstrap support. The authors note that while it may appear in this study that *Oncidium* is polyphyletic because of placement of unidentified *Oncidium* species within the *Odontoglossum* grouping and within tribe Malaxideae this is more likely a case of miss-identification (discussed later). All of the “misplaced” samples of *Oncidium* are not identified past genus, so identification was most likely attributed only on pseudobulb shape, and may be incorrect.

Oncidium heteranthum is found in different locations throughout the preserve at a high number. Concentrations of the species however are found along the walking trail up to the research station, and large clustered pockets are found in the field on the way to the river trail. Authors have noticed marked morphological differences within the species in particular how the inflorescence appears, with some species showing smaller flowers with multiple aborted flowers, and also a large variation in overall inflorescence size. This floral variation is also documented and observed by authors in Neubig et al., 2012 as a personal comment by author W.M Whitten (Neubig et al., 2012). We hypothesized that morphological variation may correlate with nucleotide variation, and speculated DNA barcoding may reveal some level of intraspecific variation. However both *matK* and *rbcL* sequences of *Oncidium heteranthum* showed very little genetic variation in the NJ trees placing species together in a cluster within the subtribe Oncidiinae. Such little variation was uncovered by the tree and the pairwise distance matrix, the authors concede for this study the idea that floral variation is indicative of a species complex, and instead see this as an example of where floral morphology is highly plastic, potentially heavily influenced by pollinator associations and should not be used to accurately depict species lines (Neubig et al., 2012, Dalstrom and Higgins, 2016).

Identification of unknown samples, further identification, and taxonomic conflicts

Results from phylogenetic, species resolution analysis, and pairwise distance matrices show that the *matK* DNA barcode is the most successful at identifying species present at the Siempre Verde Preserve. Therefore we can use the *matK* Neighbor Joining tree (and therefore the DNA Barcodes) to tentatively place unknown Orchidaceae samples into genera. Below are three such samples that authors attempted to identify via their DNA Barcode and its subsequent place in the *matK* NJ orchid phylogeny. Unknown Orchidaceae Sample number KB161 is placed on the tree with low bootstrap support (30%) within the subfamily Ependromorphae, subtribe Pleurothallidinae. Its

matK NJ tree with high BS support (87%) within the sub family Orchidoideae, tribe Cranichideae (Figure 15). Its position infers that it is a species belonging to the genus *Sauroglossum*. The pairwise distance matrix shows that unknown KB125's sequence differs to its nearest neighbor on the tree *Sauroglossum andinum* (KB 117) by 10 base pairs. Placement on the tree is in agreement with collection notes of KB125, which state that the sample is a "terrestrial with a *Sauroglossum*-like inflorescence, displaying mottled leaves that are very different in size and shape to KB117". For this sample authors should be confident in using the molecular evidence coupled with preliminary collection notes to tentatively place this sample into the genus *Sauroglossum*.

Unknown Orchidaceae Sample number KB185 was placed on the *matK* NJ tree with high bootstrap support (63%) within the sub family Epidendroideae, subtribe Oncidiinae (Figure 17). Its position infers that it is a species belonging to the genus *Odontoglossum*. Present in this clade are two identified species of *Odontoglossum* (*hallii* and *cirrhosum*) and one unidentified species of *Oncidium*. As mentioned previously in this manuscript, and also discussed later, some of the partially identified *Oncidium* species may be erroneous. The pairwise distance between the unknown sample KB185 and its nearest tree neighbor AP6945 (*Odontoglossum cirrhosum*) is zero, they are a 100% sequence match. Placement on the tree is aligned with collection notes that state the sample was very degraded with small and partial flowers present that "resembled *Odontoglossum cirrhosum* with petals and sepals removed". Authors should be confident in giving a full identification to this sample as *Odontoglossum cirrhosum* as both taxonomic and molecular identities match.

Unknown Orchidaceae Sample number KB161 is placed on the *matK* tree with low bootstrap support (30%) within the sub family Epidendroideae, subtribe Pleurothallidinae. Its

position in Clade B infers that it belongs in the genus *Stelis*. Present in the clade are two samples identified as *Stelis piperina*, and two samples of unidentified *Stelis*. If we collapsed the current clade, and looked inward toward the spine of the tree to the larger clade that displays a bootstrap value of 66, we see that the majority of samples are either unidentified *Stelis* or *Pleurothallis*, along with one sample of *Stelis pusilla*. The pairwise distance to the nearest identified neighbor on the tree *Stelis piperina* KB122 and *Stelis piperina* AP6966 are 1 and 2 nucleotides respectively. The pairwise distance to the nearest unidentified tree neighbor *Pleurothallis* sp. (KB139) is 2 nucleotides. Collection notes for this sample are limited and have no preliminary taxonomic identity. Authors cannot use the molecular information provided to make a confident identification, beyond assignment to subtribe Pleurothallidinae, because of the incongruent signals coming from the clade, where genetic distances are very similar for both *Stelis* and *Pleurothallis*.

As well as being able to infer species assignment for unidentified species the level of resolution gained in the *matK* NJ tree could guide full identification for samples placed on the tree at genus level. Several samples in subtribe Oncidiinae could not be identified past genus, many of which have been designated as *Oncidium* sp. (See Figure 19). Samples KB123 and KB121 are both positioned within the *Oncidium heteranthum* series, and are a 100% match for nearest neighbor KB201 and KB112 respectively, both of which are identified as *Oncidium heteranthum*. It is clear these samples are identified to the correct genus and it is highly probable that both samples are *Oncidium heteranthum*. Sample KB131 sits in between two species of identified *Odontoglossum* and shows 100% sequence match to sample number AP6940 *Odontoglossum hallii*. Collection notes show the sample had both floral and vegetative parts when collected but the flower stalk was immature, so identification is tenuous. It is likely that the

identification to genera *Oncidium* was incorrect, given the sequence identity and position on the tree, this sample is most likely *Odontoglossum hallii*. Lastly AP5425 identified as *Oncidium* sp. is positioned in the tribe Malaxideae, in between species of *Liparis* and *Malaxis*. This sample was taken from herbarium specimens, so no personal field collection information is available. Tribe Malaxideae contains species that are both epiphytic and terrestrial, and traditionally contain only three genera, *Liparis*, *Malaxis* and *Oberonia* (Cameron 2005). This sample cannot be identified by its position on the tree because of inconsistencies between the molecular and taxonomic identities. The herbarium sample should be checked for identification, and the sample potentially pulled from further analysis until the conflict is resolved.

Finally the *matK* NJ tree can be used to comment on placement of samples that are not congruent with current taxonomic circumscription of the Orchidaceae. For example the tree shows incorrect or dubious placement of the following samples; KB159 *Pleurothallis nivalis* positioned in tribe Cranichideae, AP5201 *Maxillaria* sp. positioned in subtribe Pleurothallidinae, AP5495 *Pleurothallis* sp. positioned in tribe Sobralieae and AP6933 *Epidendrum* sp. positioned in Oncidiinae. It is unclear why these samples display in their current positions; sampling and collection notes do not provide answers. Because most of these placements are so taxonomically erroneous, it is unlikely they are the product of miss-identification. For example *Pleurothallis nivalis* a distinctive Epidendroid epiphyte, was placed in an Orchidoid terrestrial only clade (Cranichideae). Such gross miss-identifications are unlikely, it is more likely a handling error either associated with field, lab or herbarium processing is responsible.

Future prospects

Looking to the future authors would like to continue sampling the orchid flora of the preserve, as it is estimated that this study captured over just half of the known orchid species present.

Collection trips should be planned to capitalize on different flowering phenology times, other than those already captured. Secondly serious effort should be put in during these subsequent collections to collect only material with floral and vegetative parts so that specimens can be properly identified. Many samples in this study that could not or were not identified past genus made inference from phylogenetic trees complex, such as in Clade B of subtribe Pleurothallidinae, and in areas of subtribe Oncidiinae.

Authors would also like to resample *Oncidium heteranthum* at a higher frequency and throughout its entire elevational gradient to observe any molecular differences, it is understood that this thesis provided a preliminary result that should be investigated further, and perhaps with additional molecular methods other than barcoding. The authors hope someone takes on this challenge at the research station soon.

It is also suggested that future orchid barcoding studies undertaken by the authors, seek to better understand the implications of stop codon presence in barcode sequences, primarily as current research in this area points toward a better understanding of evolutionary processes within the *matK* gene region of the Orchidaceae. Also the presence of stop codons in barcode sequences of coding regions can be grounds for non-barcode compliant sequences on BOLD, and even reduce the use or potentially totally eliminate sequences from research data sets.

Lastly, to better resolve taxonomic complex genera in subtribe Pleurothallidinae additional loci need to be tested, and further research focus should be given to untangling Clade B and its possible correlation to the subtribe *Crocodelanthae*. This is particularly pertinent to

gaining a clearer understanding of the genetic relationships between *Pleurothallis* and *Stelis* and other subgeneric groupings within the two genera. It is clear from this study, *matK* is efficient at handling many of the species in the orchid flora of SV, however future work in subtribe Pleurothallidinae should look at less traditional barcodes such as *trnH-psbA* or *ITS*, both of which have shown to work well in Orchidaceae.

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Figures



Figure 1. Trail map of Siempre Verde with topographical map

Figure 2. Total number of samples collected, and number of unique species per genera for taxon collected at Siempre Verde. This graph excludes samples collected in target genera *Pleurothallis*, *Epidendrum*, *Stelis*, *Lepanthes* and *Oncidium*. Genera with no identified species are marked as zero.

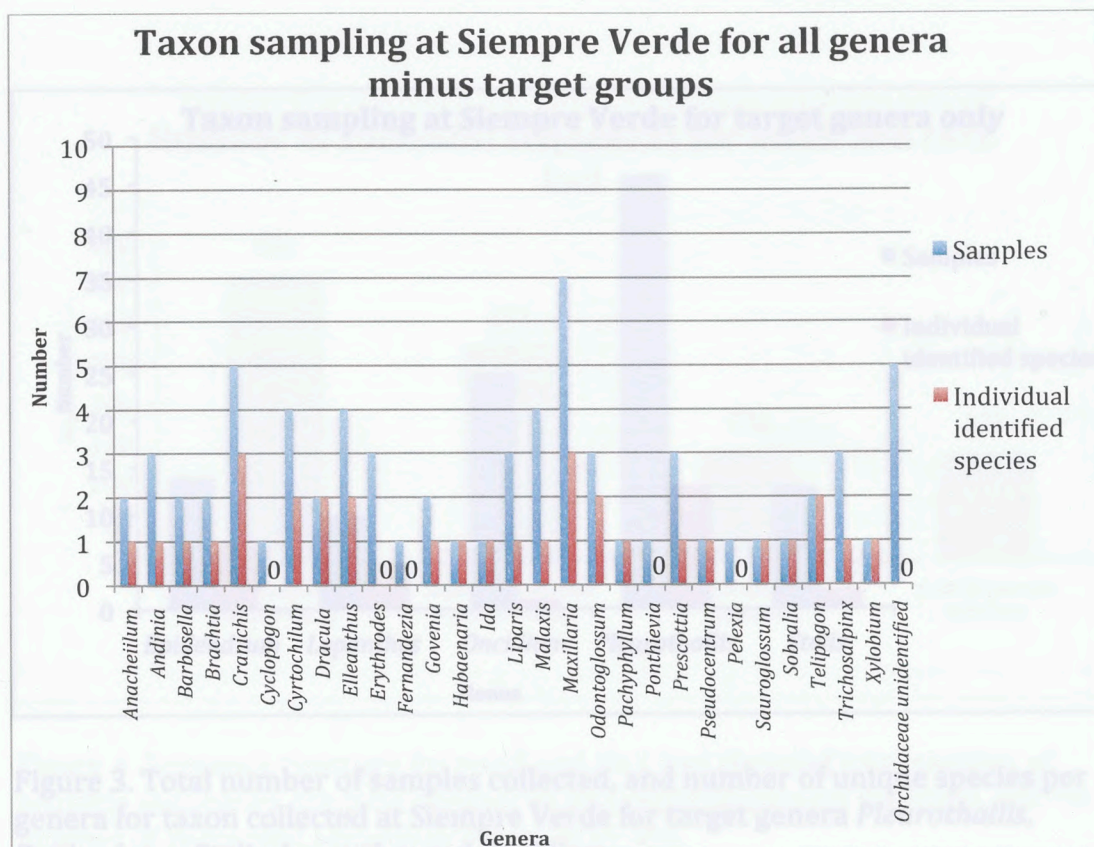


Figure 2. Total number of samples collected, and number of unique species per genera for taxon collected at Siempre Verde. This graph excludes samples collected in target genera *Pleurothallis*, *Epidendrum*, *Stelis*, *Lepanthes* and *Oncidium*. Genera with no identified species are marked as zero.

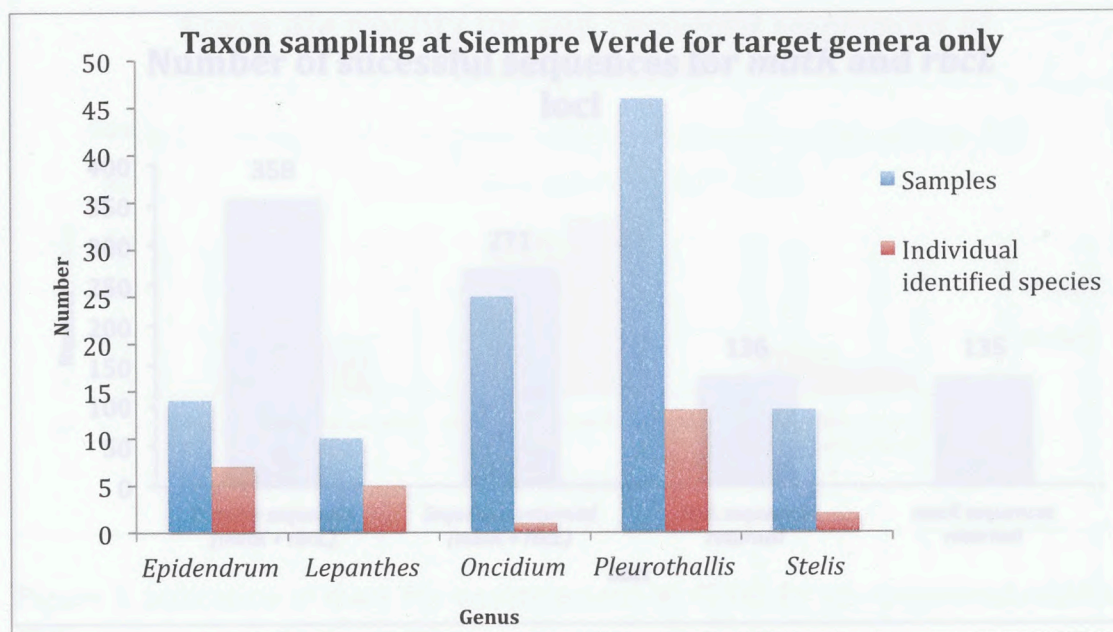


Figure 3. Total number of samples collected, and number of unique species per genera for taxon collected at Siempre Verde for target genera *Pleurothallis*, *Epidendrum*, *Stelis*, *Lepanthes* and *Oncidium*.

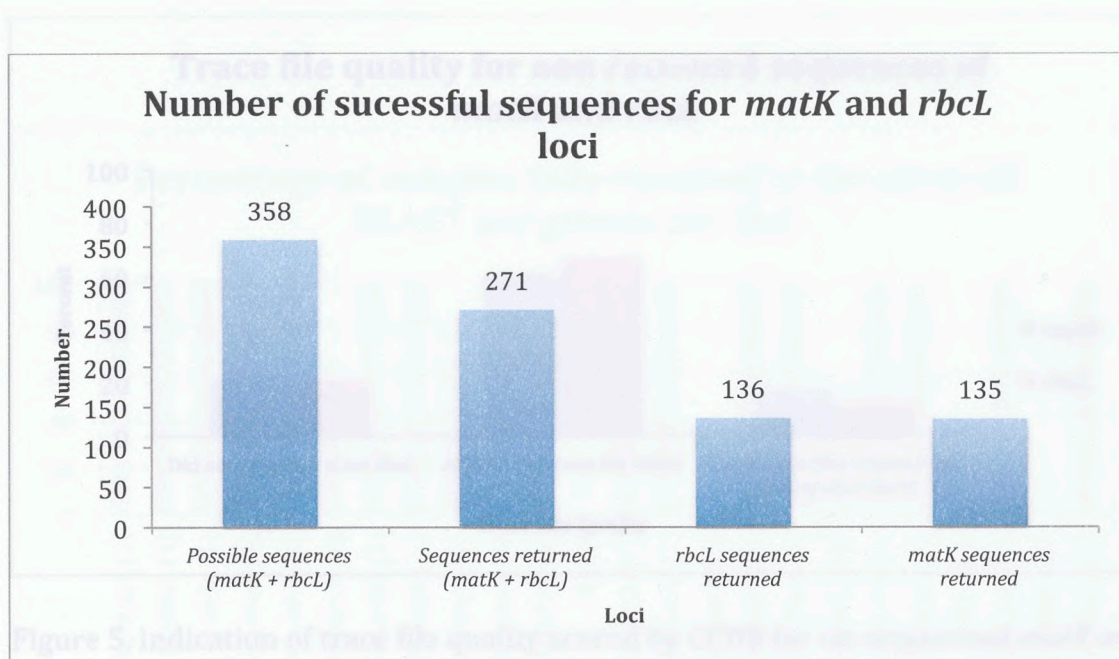


Figure 4. Sequence recovery for *matK* and *rbcL* loci showing total number of possible sequences, the total number of successful sequences returned, and the number of successful sequences returned by loci.

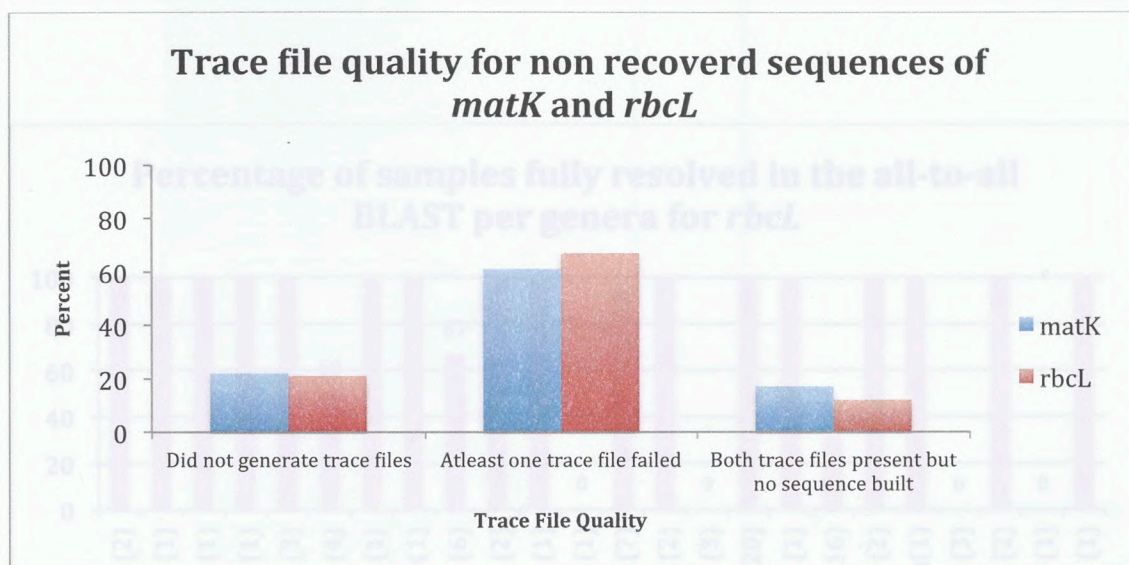


Figure 5. Indication of trace file quality scored by CCDB for un-sequenced *matK* and *rbcl*.

Figure 6. Percentage of samples fully resolved by the all-to-all BLAST shown per genus for *rbcl* loci. Numbers in parenthesis after genus indicates total number of samples in the analysis. Genera with less than 100% resolution were given data labels to show percentage.

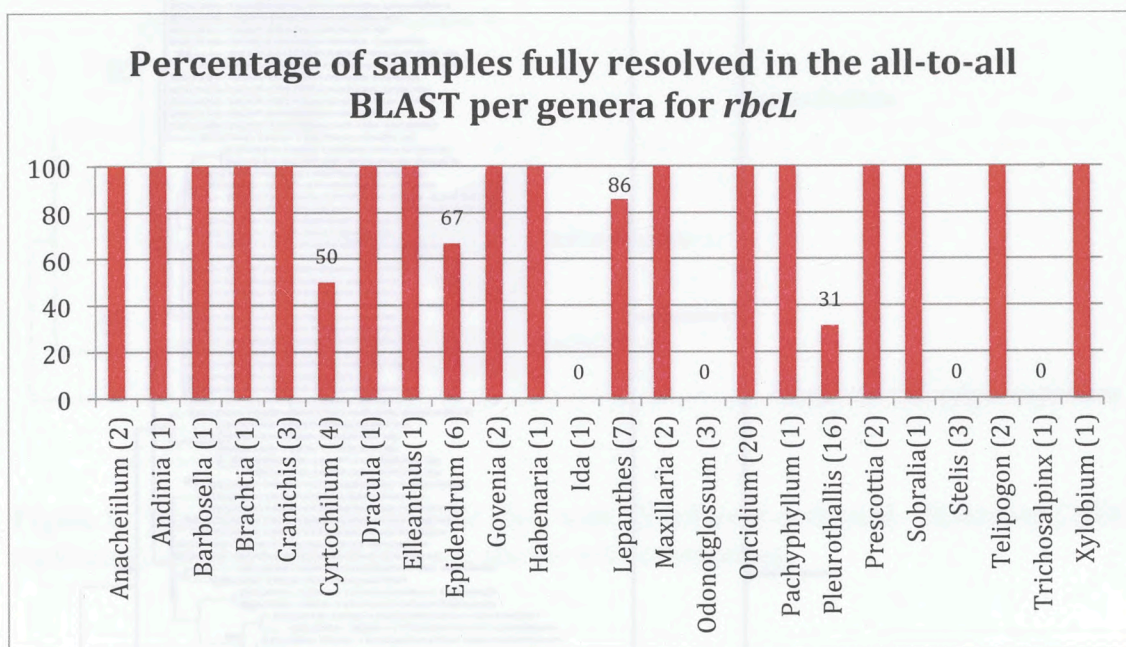


Figure 6. Percentage of samples fully resolved by the all-to-all BLAST shown per genus for *rbcl* loci. Numbers in parenthesis after genus indicates total number of samples in the analysis. Genera with less than 100% resolution were given data labels to show percentage.

Figure 7. Neighbor Joining Tree for *rbcl*, bootstrap (2000 replicates) support values >50% are shown next to branches.

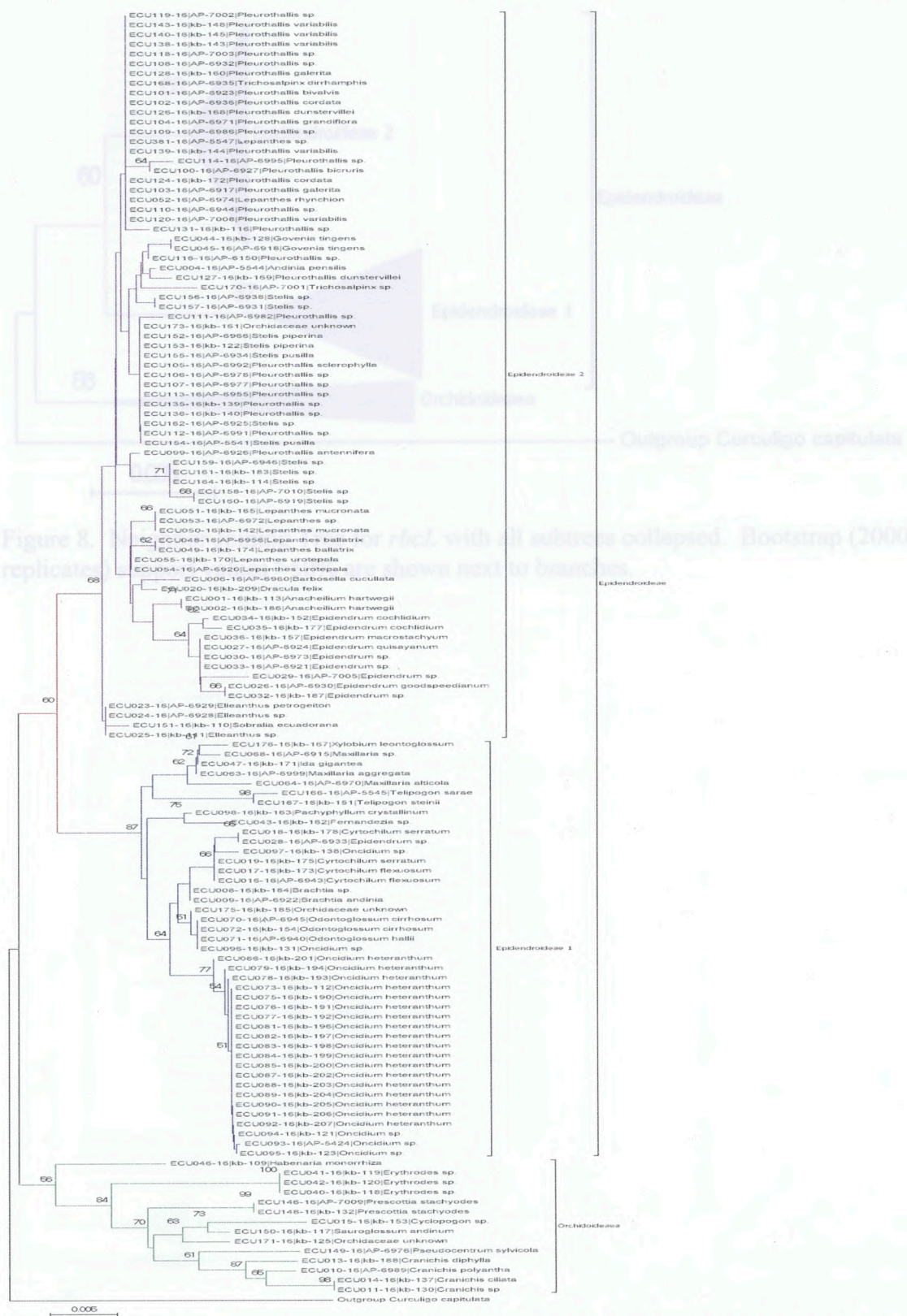


Figure 7. Neighbor Joining Tree for *rbcL*, bootstrap (2000 replicates) support values >50% are shown next to branches.

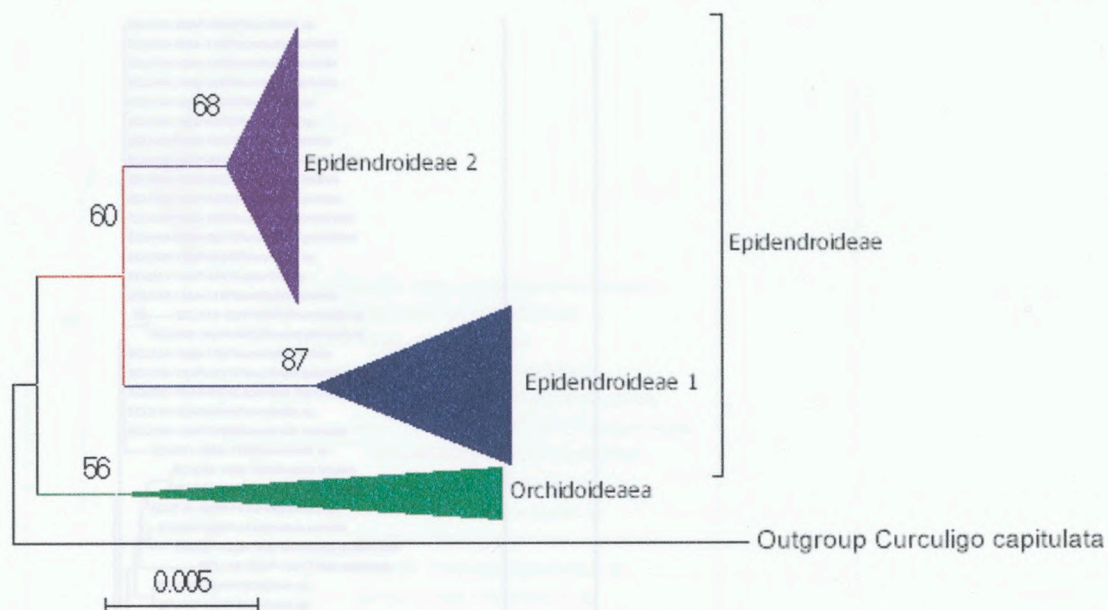


Figure 8. Neighbor Joining Tree for *rbcL* with all subtrees collapsed. Bootstrap (2000 replicates) support values >50% are shown next to branches.



Figure 9. Neighbor Joining Tree for *rbcL* with subtrees collapsed for subfamily Orchidoideae, and Epidendroideae 1.

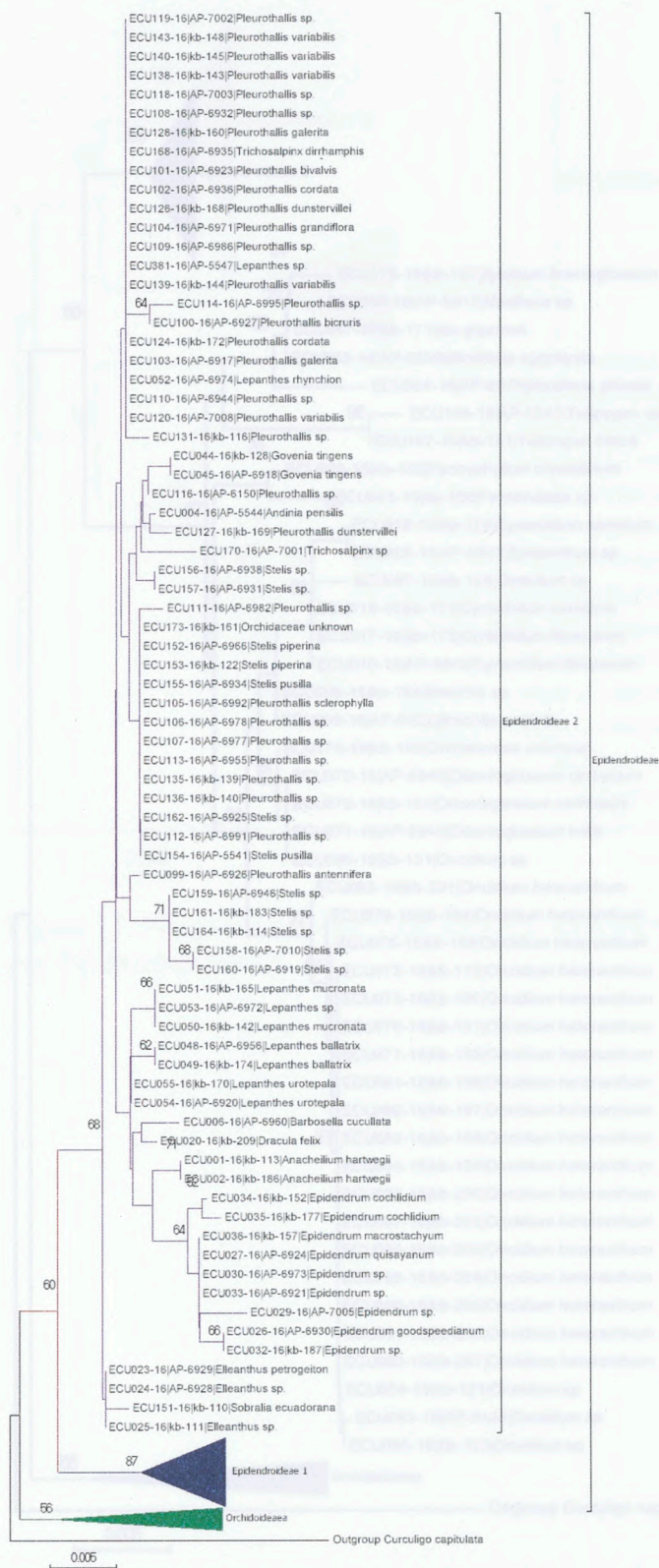


Figure 9. Neighbor Joining Tree for *rbcL* with subtrees collapsed for sub family Orchidoideae, and Epidendroideae 1.

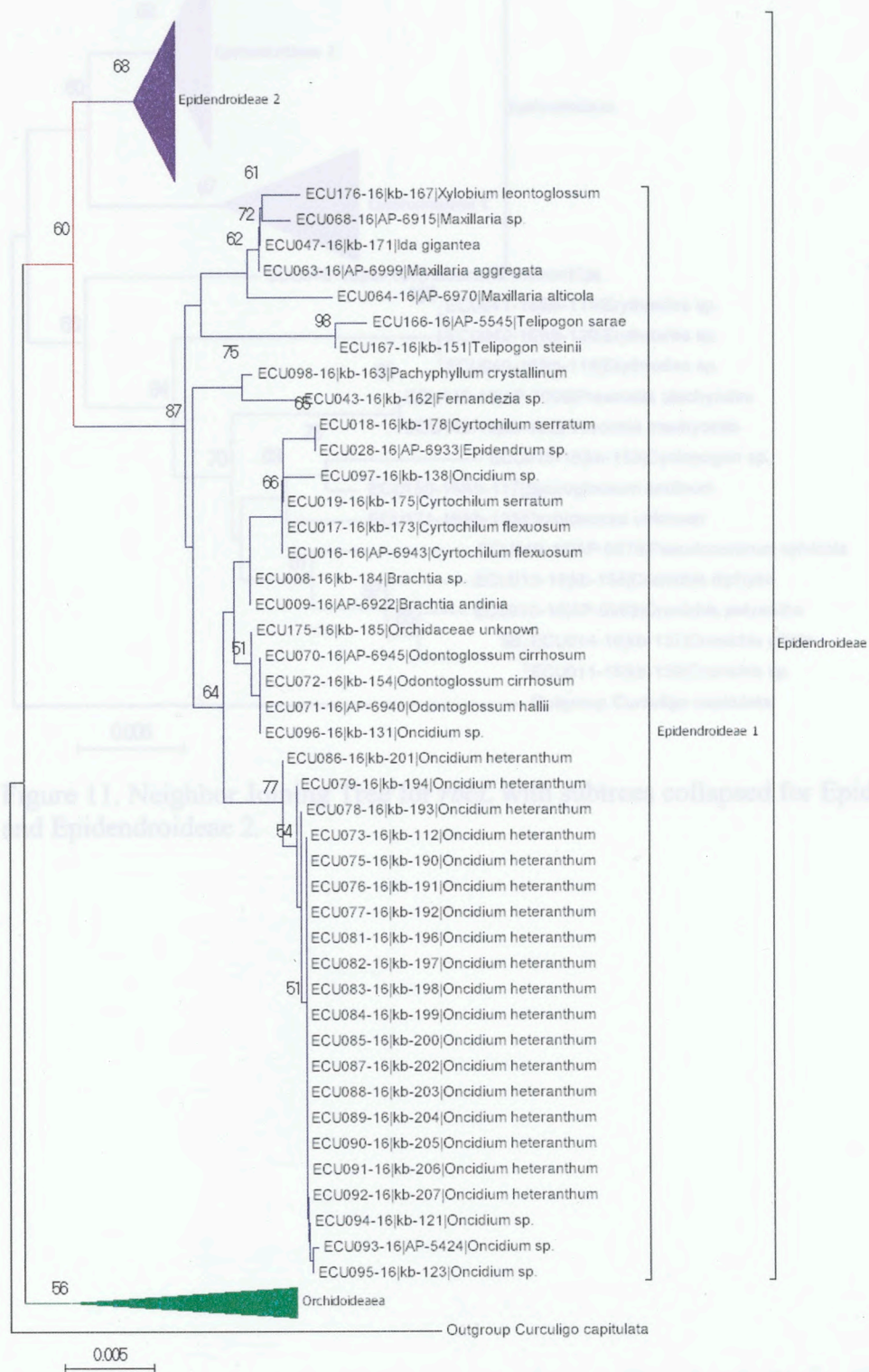


Figure 10. Neighbor Joining Tree for *rbcL* with subtrees collapsed for sub family Orchidoideae, and Epidendroideae 2.

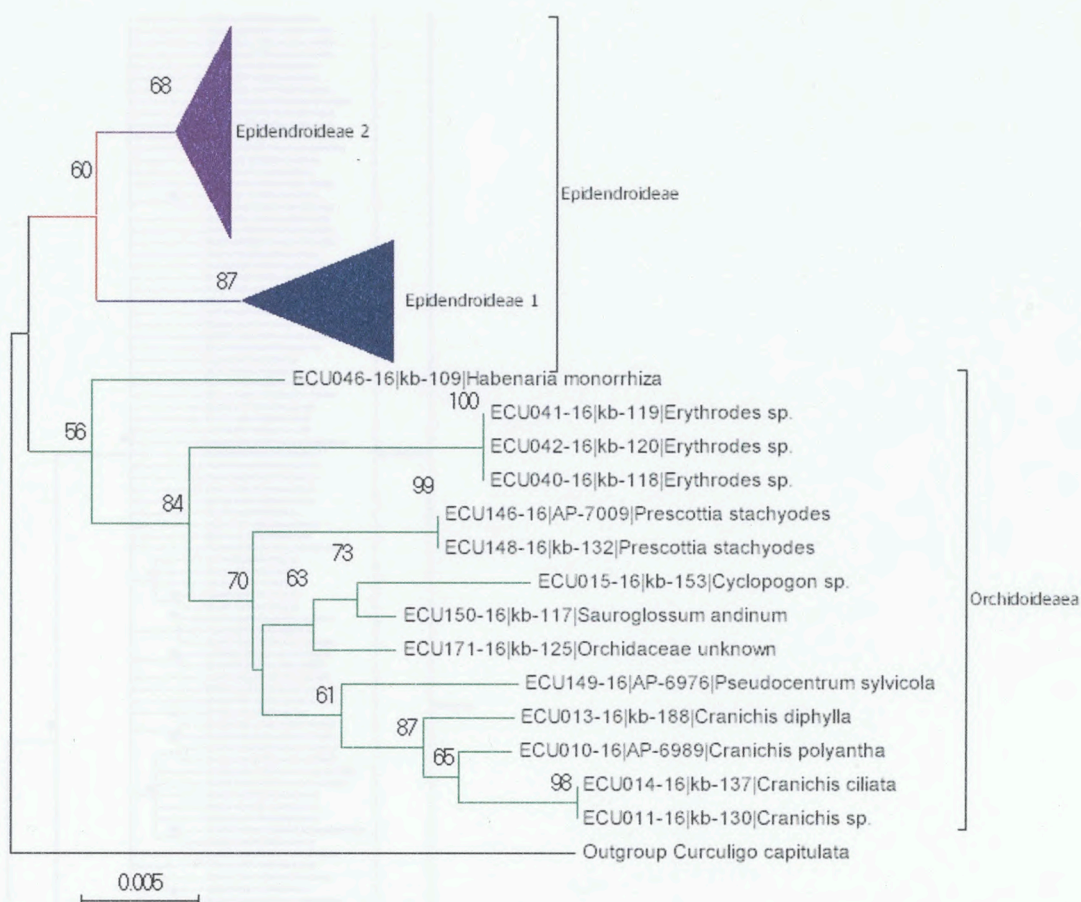


Figure 11. Neighbor Joining Tree for *rbcL* with subtrees collapsed for Epidendroideae 1 and Epidendroideae 2.

Figure 12. Neighbor Joining *rbcL* map showing only the branches that have <50% bootstrap support are collapsed.

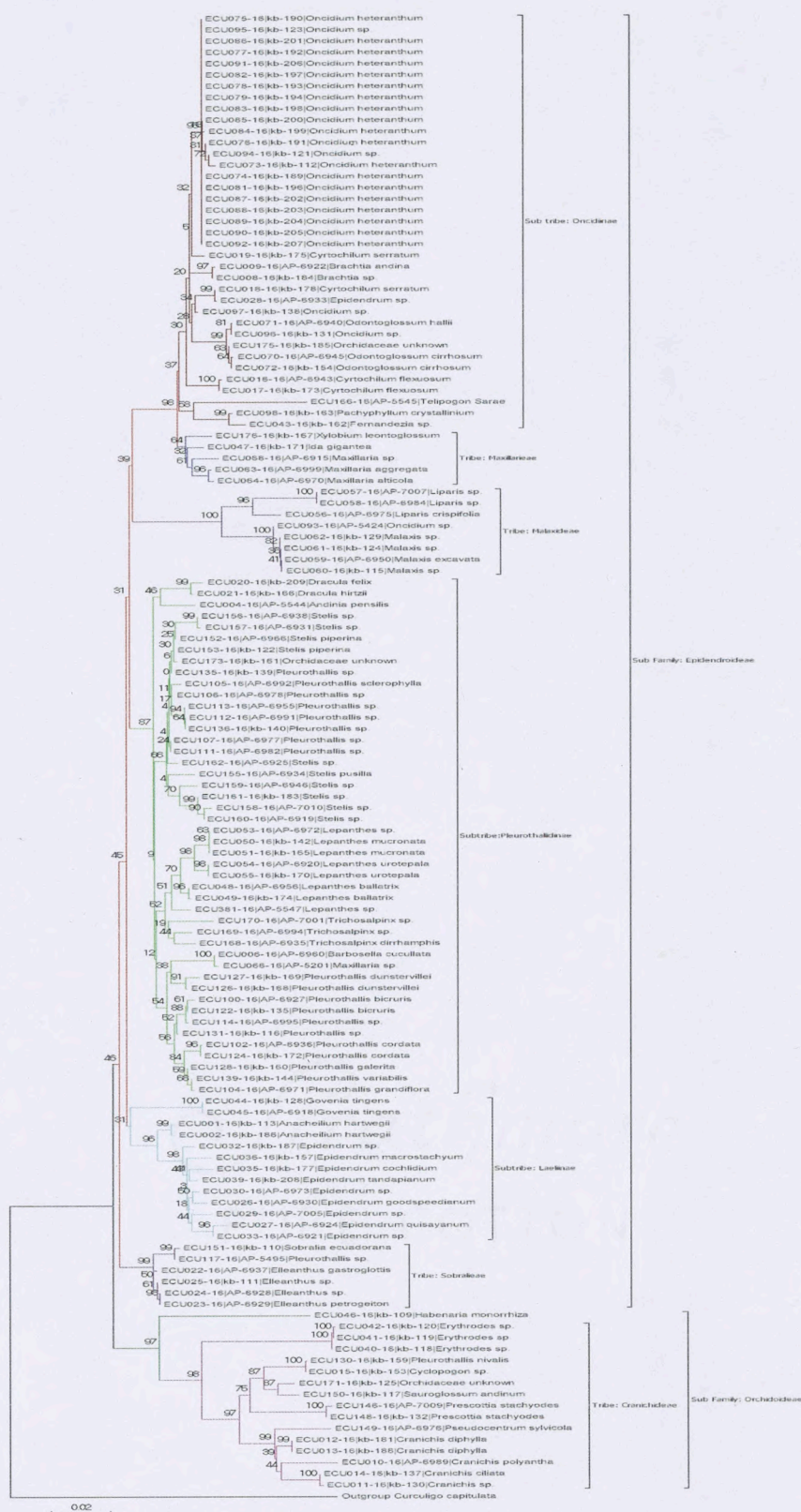


Figure 13. Neighbor Joining Tree for *matK*. bootstrap (2000 replicates) support values are shown next to branches.

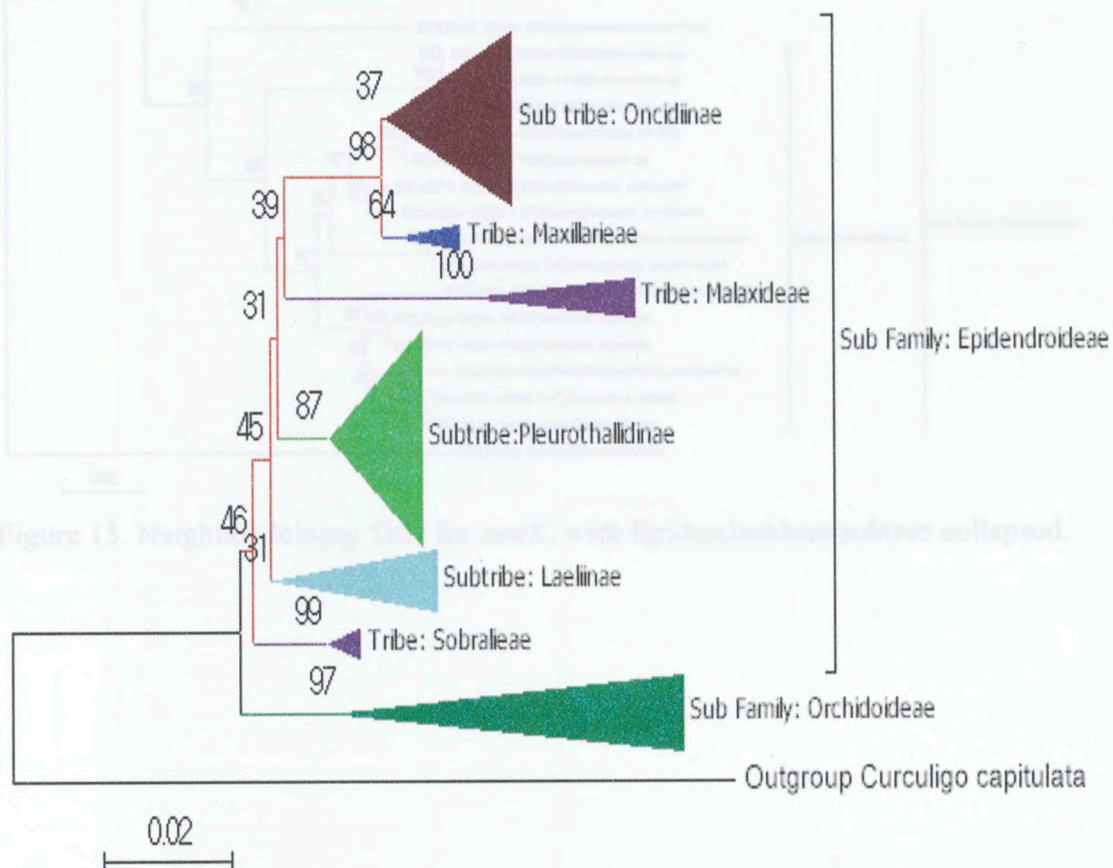


Figure 14. Neighbor Joining Tree for *matK* with all subtrees collapsed.

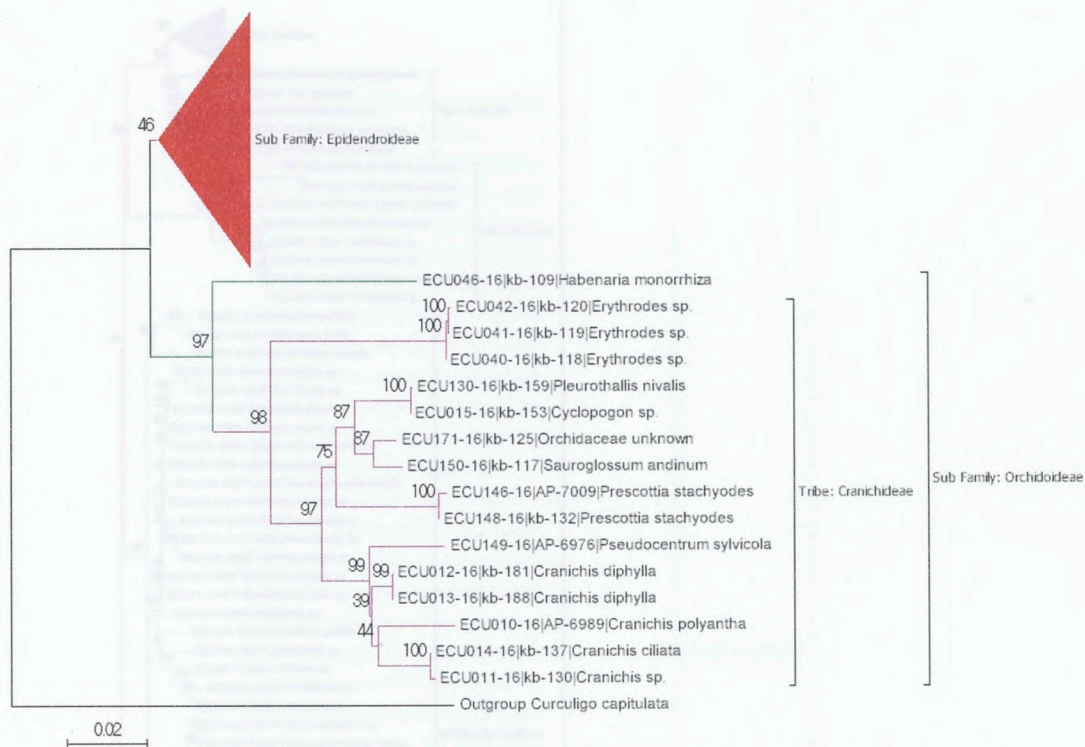


Figure 15. Neighbor Joining Tree for *matK*, with Epidendroideae subtree collapsed.

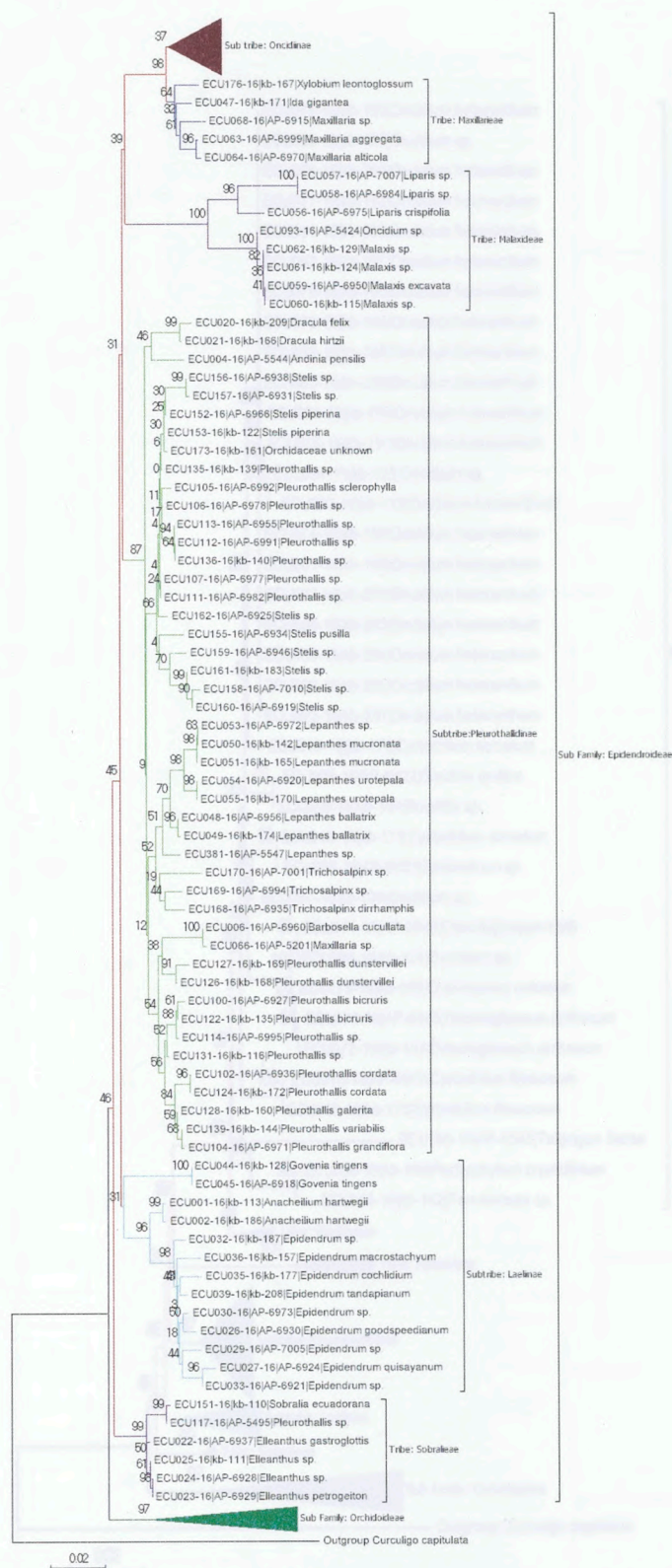


Figure 16. Neighbor Joining tree for *matK* with subtrees Orchidoideae and Oncidiinae collapsed.

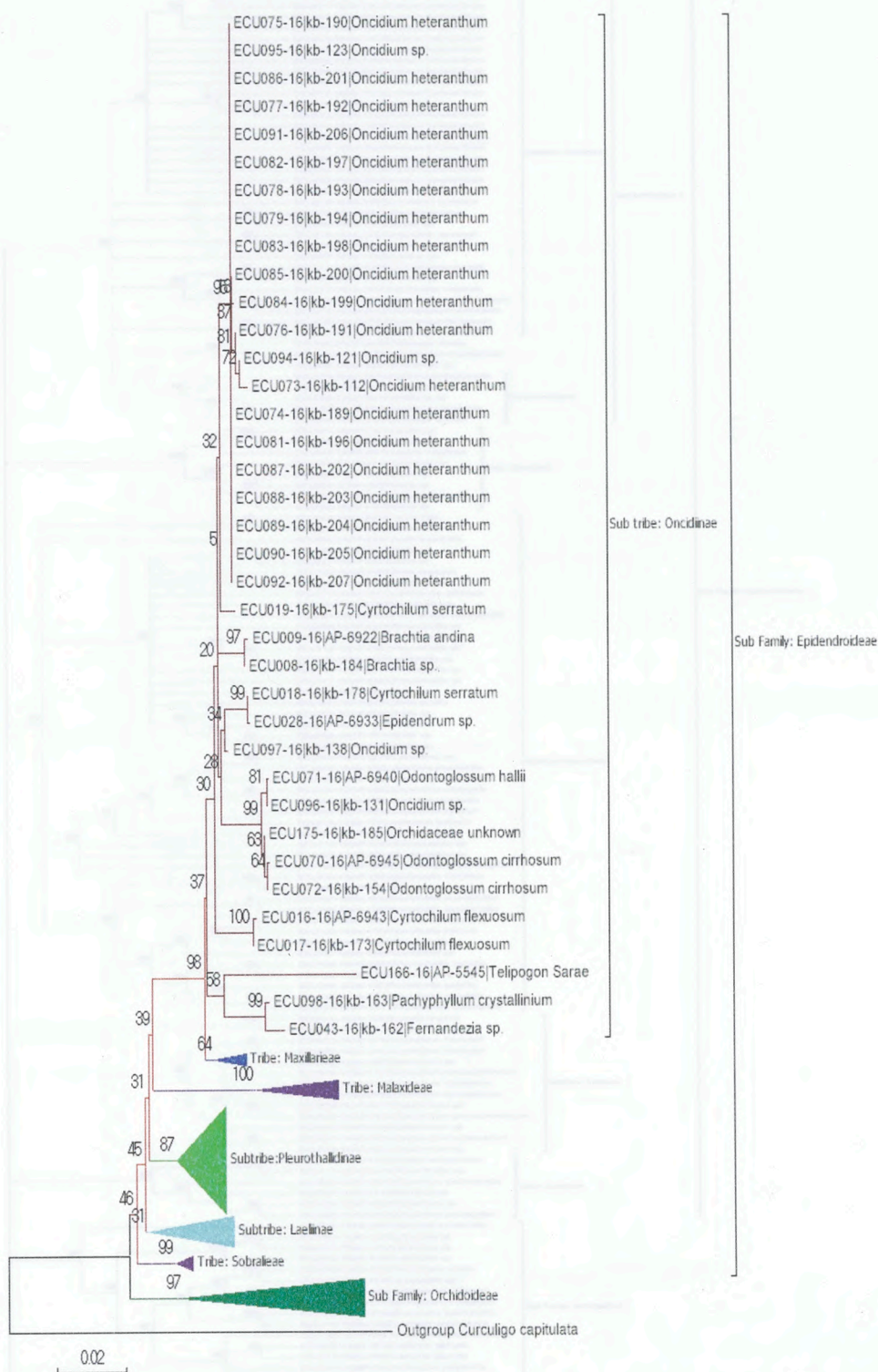


Figure 17. Neighbor Joining tree for *matK* with subtrees collapsed to show detail for subtribe Oncidiinae.

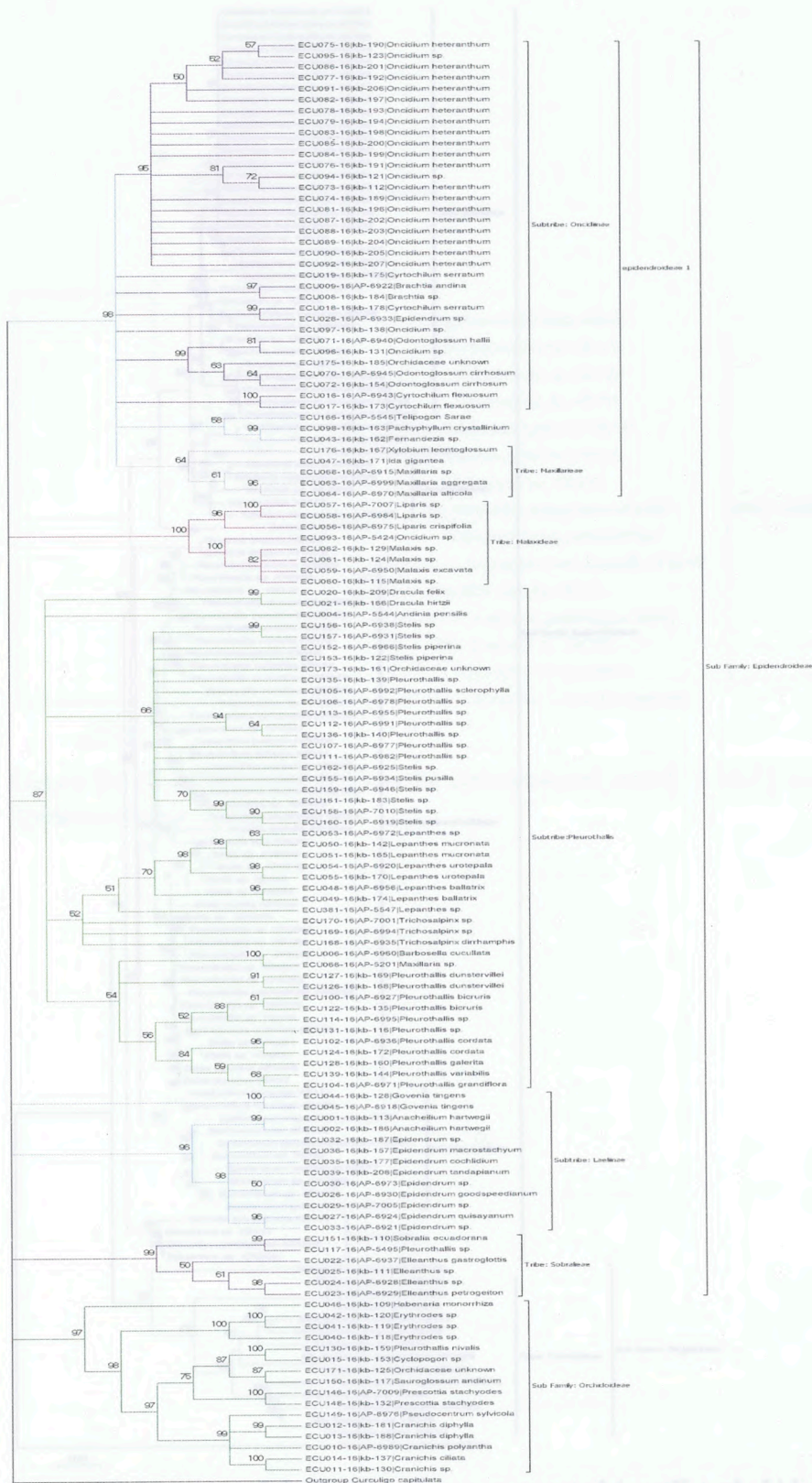


Figure 18. Neighbor Joining *matK* majority rule tree. Branches that have <50% bootstrap support are collapsed.

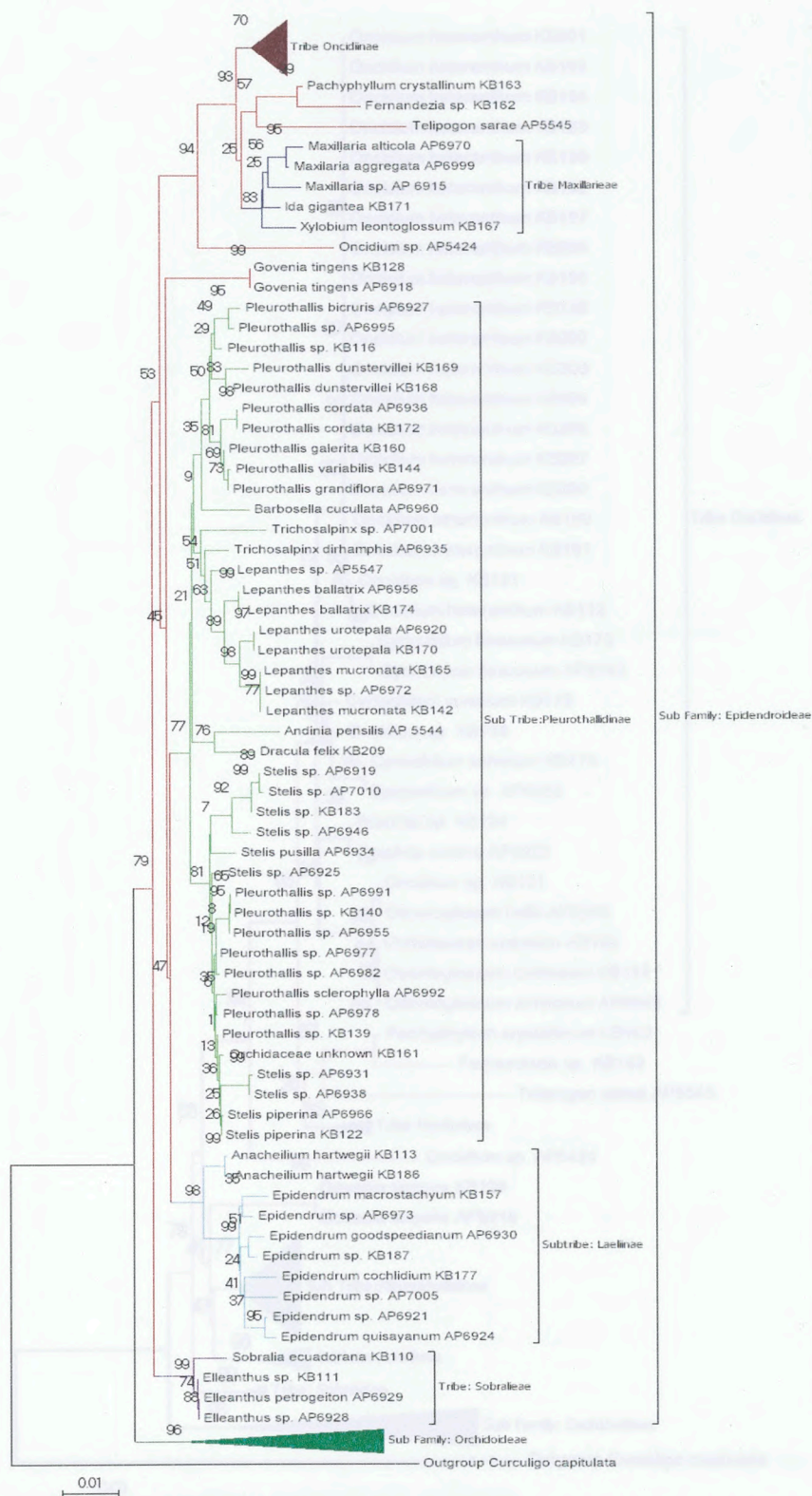


Figure 21. Neighbor Joining Tree for concatenated (*matK* + *rbcL*) with subtrees Orchidoideae and Oncidiinae.

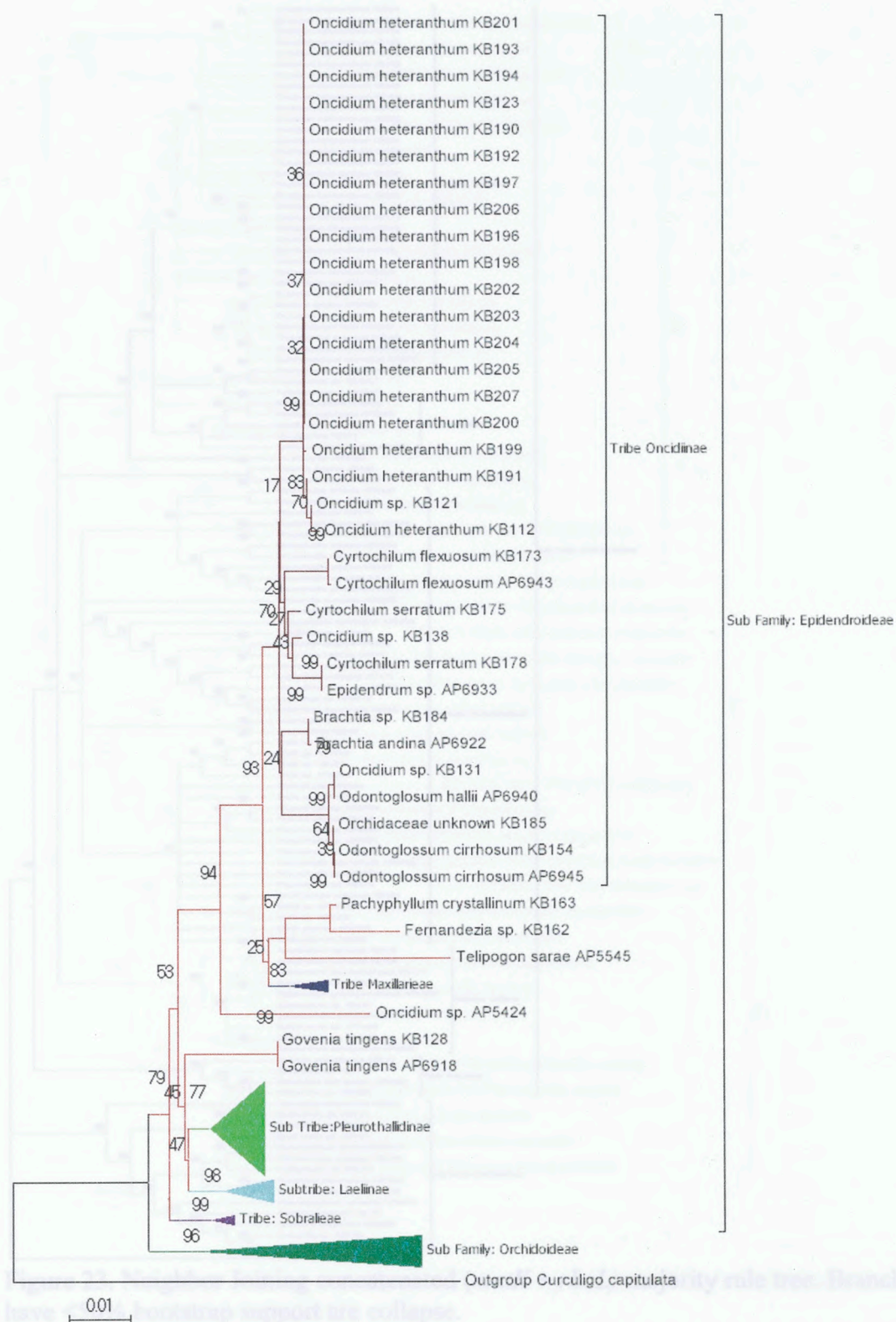


Figure 22. Neighbor Joining Tree for concatenated (*matK* + *rbcL*) showing subtree Oncidiinae.

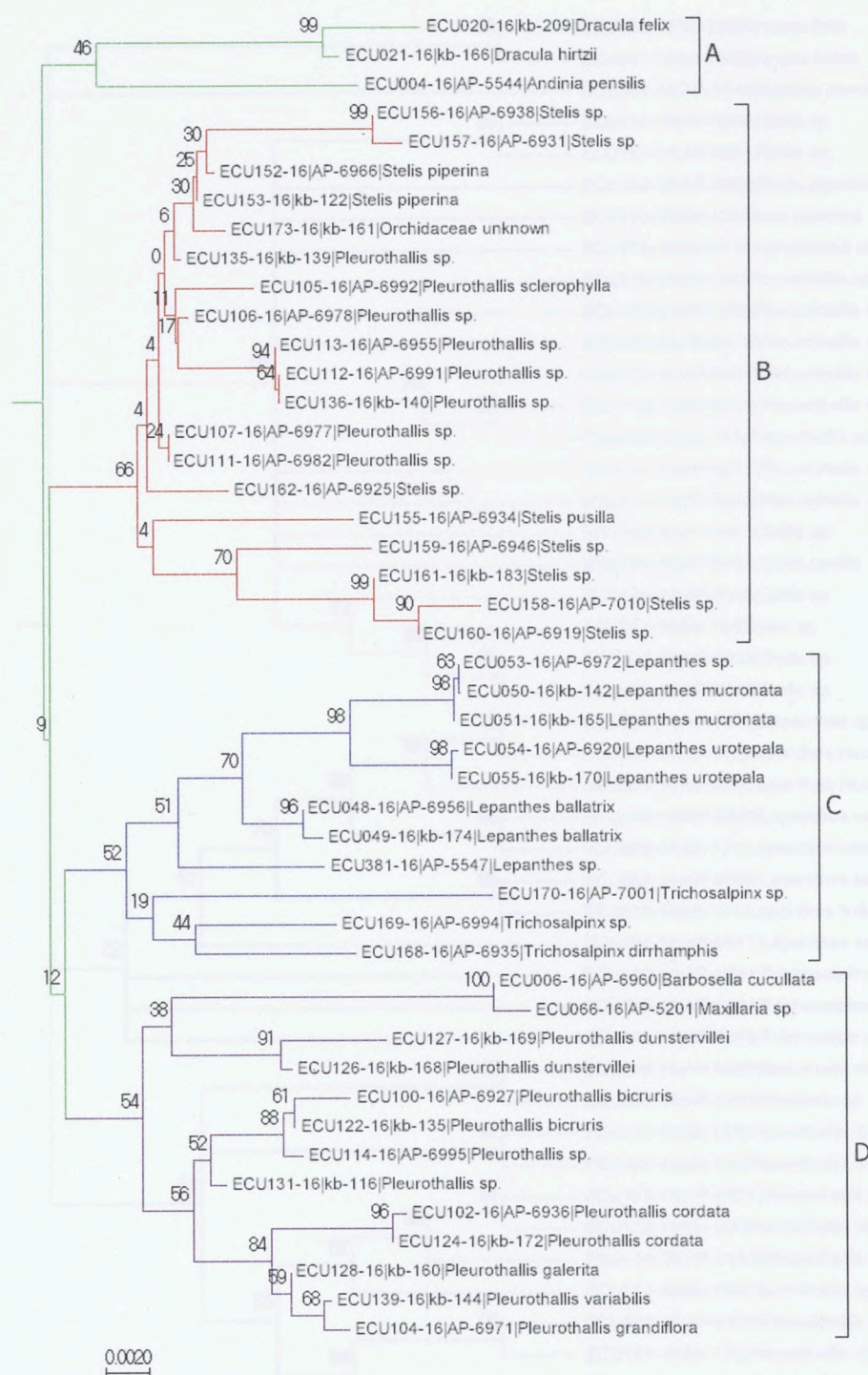


Figure 24. Neighbor Joining tree for *matK* showing sub tree Pleurothallidinae in detail with 4 major clades labeled A-D.

Figure 25. Neighbor Joining tree Majority Rule tree for *matK* showing only sub tree Pleurothallidinae. Branches with <50% bootstrap support have been collapsed.

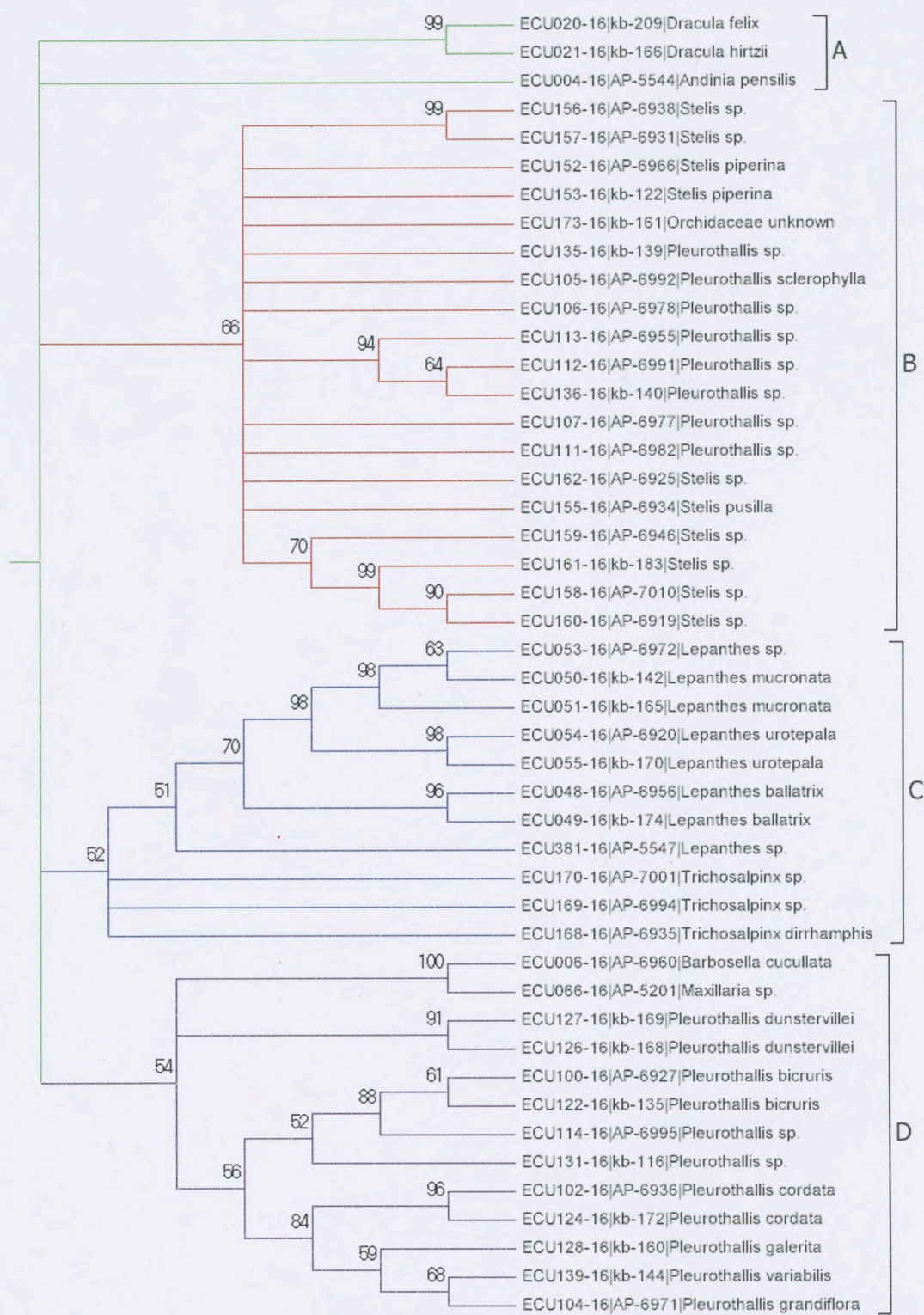


Figure 25. Neighbor Joining tree Majority Rule tree for *matK* showing only sub tree Pleurothallidinae. Branches with <50% Bootstrap support have been collapsed.

Table1. Primer sets used for replication of *matK* and *rbcL* at CCDB during replication.

rbcLa-F	ATGTCACCACAAACAGAGACTAAAGC	Levin et al, 2003
rbcLa-R	GTAAATCAAGTCCACCRG	Kress & Erickson, 2009

Forward: matK-xf	TAATTTACGATCAATTCATTC	Ford et al. 2009
Reverse: matK-MALP	ACAAGAAAGTCGAAGTAT	Dunning & Savolainen, 2010

Accession	Species	Family	Order	Class	Phylum	Reference
U00001.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	van der Meer et al. 2008
U00002.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00003.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00004.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00005.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00006.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00007.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00008.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00009.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00010.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00011.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00012.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00013.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00014.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00015.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00016.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00017.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00018.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00019.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00020.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00021.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00022.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00023.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00024.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009
U00025.10	<i>Arabidopsis</i>	<i>Brassicaceae</i>	<i>Brassicales</i>	<i>Equisetum</i>	<i>Embryophyta</i>	Prigmore et al. 2009

Table 2. Samples collected from the Siempre Verde preserve in Imbabura, Ecuador. Barcode Of Life Database process ID, Taxonomic identification including subfamily, tribe and subtribe and reference for taxonomic placement.

BOLD Process ID	Genus	Species	Subfamily	Tribe	Subtribe	Reference
ECU001-16 ^b	<i>Anacheilum</i>	<i>hartwegii</i>	Epidendroideae	Epidendreae	Laeliinae	van den Berg et al., 2009
ECU002-16 ^b	<i>Anacheilum</i>	<i>hartwegii</i>	Epidendroideae	Epidendreae	Laeliinae	van den Berg et al., 2009
ECU003-16 ^a	<i>Andinia</i>	<i>pensilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Pridgeon et al., 2001
ECU004-16 ^{a,b}	<i>Andinia</i>	<i>pensilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Pridgeon et al., 2001
ECU005-16 ^a	<i>Andinia</i>	<i>pensilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Pridgeon et al., 2001
ECU006-16 ^b	<i>Barbosella</i>	<i>cuculata</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU007-16	<i>Barbosella</i>	<i>cuculata</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU008-16 ^b	<i>Bractia</i>	<i>sp.</i>	Epidendroideae	Cymbidieae	Oncidiinae	Pridgeon et al., 2014
ECU009-16 ^b	<i>Bractia</i>	<i>andina</i>	Epidendroideae	Cymbidieae	Oncidiinae	Pridgeon et al., 2014
ECU010-16 ^b	<i>Cranichis</i>	<i>polyantha</i>	Orchidoideae	Cranichideae	Cranichidinae	Salazar et al. 2009
ECU011-16 ^b	<i>Cranichis</i>	<i>sp.</i>	Orchidoideae	Cranichideae	Cranichidinae	Salazar et al. 2009
ECU012-16	<i>Cranichis</i>	<i>diphylla</i>	Orchidoideae	Cranichideae	Cranichidinae	Salazar et al. 2009
ECU013-16 ^b	<i>Cranichis</i>	<i>diphylla</i>	Orchidoideae	Cranichideae	Cranichidinae	Salazar et al. 2009
ECU014-16 ^b	<i>Cranichis</i>	<i>ciliata</i>	Orchidoideae	Cranichideae	Cranichidinae	Salazar et al. 2009
ECU015-16 ^b	<i>Cyclopogon</i>	<i>sp.</i>	Orchidoideae	Cranichideae	cyclopogoninae	Górniak et al. 2006
ECU016-16 ^b	<i>Cyrtorchilum</i>	<i>flexuosum</i>	Epidendroideae	Cymbidieae	Oncidiinae	Williams et al., 2001
ECU017-16 ^b	<i>Cyrtorchilum</i>	<i>flexuosum</i>	Epidendroideae	Cymbidieae	Oncidiinae	Williams et al., 2001
ECU018-16 ^b	<i>Cyrtorchilum</i>	<i>serratum</i>	Epidendroideae	Cymbidieae	Oncidiinae	Williams et al., 2001
ECU019-16 ^b	<i>Cyrtorchilum</i>	<i>serratum</i>	Epidendroideae	Cymbidieae	Oncidiinae	Williams et al., 2001
ECU020-16 ^b	<i>Dracula</i>	<i>felix</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU021-16	<i>Dracula</i>	<i>hirtzii</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU024-16 ^b	<i>Elleanthus</i>	<i>sp.</i>	Epidendroideae	Sobralieae		Neubig et al., 2011
ECU022-16	<i>Elleanthus</i>	<i>gastroglottis</i>	Epidendroideae	Sobralieae		Neubig et al., 2011
ECU023-16 ^b	<i>Elleanthus</i>	<i>petrogeiton</i>	Epidendroideae	Sobralieae		Neubig et al., 2011
ECU025-16 ^b	<i>Elleanthus</i>	<i>sp.</i>	Epidendroideae	Sobralieae		Neubig et al., 2011

ECU028-16 ^b	<i>Epidendrum</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU033-16 ^b	<i>Epidendrum</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU026-16 ^b	<i>Epidendrum</i>	<i>goodspeedianum</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU027-16 ^b	<i>Epidendrum</i>	<i>quisayanum</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU029-16 ^b	<i>Epidendrum</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU030-16 ^b	<i>Epidendrum</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU031-16 ^a	<i>Epidendrum</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU032-16 ^b	<i>Epidendrum</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU034-16 ^b	<i>Epidendrum</i>	<i>cochlidium</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU035-16 ^b	<i>Epidendrum</i>	<i>cochlidium</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU038-16	<i>Epidendrum</i>	<i>geminiflorum</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU036-16 ^b	<i>Epidendrum</i>	<i>macrostachyum</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU037-16	<i>Epidendrum</i>	<i>mancum</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU039-16	<i>Epidendrum</i>	<i>tandapianum</i>	Epidendroideae	Epidendreae	Laeliinae	Dressler (1993)
ECU040-16 ^b	<i>Erythroides</i>	<i>sp.</i>	Orchidoideae	Cranichideae	Goodyearinae	Hu et al., 2016
ECU041-16 ^b	<i>Erythroides</i>	<i>sp.</i>	Orchidoideae	Cranichideae	Goodyearinae	Hu et al., 2016
ECU042-16 ^b	<i>Erythroides</i>	<i>sp.</i>	Orchidoideae	Cranichideae	Goodyearinae	Hu et al., 2016
ECU044-16 ^b	<i>Govenia</i>	<i>tingens</i>	Epidendroideae	Cymbideae	Goveninae	Dressler (1993)
ECU045-16 ^b	<i>Govenia</i>	<i>tingens</i>	Epidendroideae	Cymbideae	Goveninae	Dressler (1993)
ECU046-16 ^b	<i>Habaenaria</i>	<i>monorrhiza</i>	Orchidoideae	Orchideae	Habenariinae	Dressler (1993)
ECU047-16 ^b	<i>Ida</i>	<i>gigantea</i>	Epidendroideae	Cymbideae	Maxillariinae	Dressler (1993)
ECU048-16 ^b	<i>Lepanthes</i>	<i>ballatrix</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU049-16 ^b	<i>Lepanthes</i>	<i>ballatrix</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU380-16 ^a	<i>Lepanthes</i>	<i>columbar</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU050-16 ^b	<i>Lepanthes</i>	<i>mucronata</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU051-16 ^b	<i>Lepanthes</i>	<i>mucronata</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU052-16 ^b	<i>Lepanthes</i>	<i>rhynchion</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU053-16 ^b	<i>Lepanthes</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU381-16 ^a ^b	<i>Lepanthes</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU054-16 ^b	<i>Lepanthes</i>	<i>urotepala</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU055-16 ^b	<i>Lepanthes</i>	<i>urotepala</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)

ECU057-16	<i>Liparis</i>	<i>sp.</i>	Epidendroideae	Malaxideae		Cameron (2005)
ECU058-16	<i>Liparis</i>	<i>sp.</i>	Epidendroideae	Malaxideae		Cameron (2005)
ECU056-16	<i>Liparis</i>	<i>crispifolia</i>	Epidendroideae	Malaxideae		Cameron (2005)
ECU060-16	<i>Malaxis</i>	<i>sp.</i>	Epidendroideae	Malaxideae		Cameron (2005)
ECU061-16	<i>Malaxis</i>	<i>sp.</i>	Epidendroideae	Malaxideae		Cameron (2005)
ECU062-16	<i>Malaxis</i>	<i>sp.</i>	Epidendroideae	Malaxideae		Cameron (2005)
ECU059-16	<i>Malaxis</i>	<i>excavata</i>	Epidendroideae	Malaxideae		Cameron (2005)
ECU063-16 ^b	<i>Maxillaria</i>	<i>aggregata</i>	Epidendroideae	Cymbideae	Maxillarinae	Whitten et al., 2007
ECU064-16 ^b	<i>Maxillaria</i>	<i>alticola</i>	Epidendroideae	Cymbideae	Maxillarinae	Whitten et al., 2007
ECU065-16 ^a	<i>Maxillaria</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Maxillarinae	Whitten et al., 2007
ECU066-16 ^a	<i>Maxillaria</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Maxillarinae	Whitten et al., 2007
ECU067-16	<i>Maxillaria</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Maxillarinae	Whitten et al., 2007
ECU068-16 ^b	<i>Maxillaria</i>	<i>Sp.</i>	Epidendroideae	Cymbideae	Maxillarinae	Whitten et al., 2007
ECU069-16	<i>Maxillaria</i>	<i>alticola</i>	Epidendroideae	Cymbideae	Maxillarinae	Whitten et al., 2007
ECU070-16 ^b	<i>Odontoglossum</i>	<i>cirrhosum</i>	Epidendroideae	Cymbideae	Oncidiinae	Williams et al., 2001
ECU071-16 ^b	<i>Odontoglossum</i>	<i>hallii</i>	Epidendroideae	Cymbideae	Oncidiinae	Williams et al., 2001
ECU072-16 ^b	<i>Odontoglossum</i>	<i>cirrhosum</i>	Epidendroideae	Cymbideae	Oncidiinae	Williams et al., 2001
ECU073-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU074-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU075-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU076-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU077-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU078-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU079-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU080-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU081-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU082-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU083-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU084-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU085-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU086-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU087-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012

ECU088-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU089-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU090-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU091-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU092-16 ^b	<i>Oncidium</i>	<i>heteranthum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU093-16 ^{a,b}	<i>Oncidium</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU094-16 ^b	<i>Oncidium</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU095-16 ^b	<i>Oncidium</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU096-16 ^b	<i>Oncidium</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU097-16 ^b	<i>Oncidium</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU043-16 ^b	<i>Fernandezia</i>	<i>sp.</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU098-16 ^b	<i>Pachyphyllum</i>	<i>crystallinum</i>	Epidendroideae	Cymbideae	Oncidiinae	Neubig et al., 2012
ECU150-16 ^b	<i>Sauroglossum</i>	<i>andinum</i>	Orchidoideae	Cranichidea	Spiranthinae	Górniak et al. 2006
ECU099-16	<i>Pleurothallis</i>	<i>antennifera</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU100-16	<i>Pleurothallis</i>	<i>bicruris</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU101-16	<i>Pleurothallis</i>	<i>bivalvis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU108-16	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU118-16	<i>Pleurothallis</i>	<i>Sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU119-16	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU109-16	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU110-16	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU111-16 ^b	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU112-16 ^b	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU113-16 ^b	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU102-16 ^b	<i>Pleurothallis</i>	<i>cordata</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU103-16	<i>Pleurothallis</i>	<i>galerita</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU104-16 ^b	<i>Pleurothallis</i>	<i>grandiflora</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU105-16 ^b	<i>Pleurothallis</i>	<i>sclerophylla</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU106-16 ^b	<i>Pleurothallis</i>	<i>sp</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU107-16 ^b	<i>Pleurothallis</i>	<i>sp</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU114-16 ^b	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU115-16 ^a	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)

ECU116-16 ^a	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU117-16 ^a	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU120-16	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU121-16	<i>Pleurothallis</i>	<i>bicuris</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU122-16	<i>Pleurothallis</i>	<i>bicuris</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU123-16	<i>Pleurothallis</i>	<i>bicuris</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU124-16 ^b	<i>Pleurothallis</i>	<i>cordata</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU125-16	<i>Pleurothallis</i>	<i>cordata</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU126-16 ^b	<i>Pleurothallis</i>	<i>dunstervillei</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU127-16 ^b	<i>Pleurothallis</i>	<i>dunstervillei</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU128-16 ^b	<i>Pleurothallis</i>	<i>galerita</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU129-16	<i>Pleurothallis</i>	<i>gracillima</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU130-16	<i>Pleurothallis</i>	<i>nivalis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU131-16 ^b	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU132-16	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU133-16	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU134-16	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU135-16 ^b	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU136-16 ^b	<i>Pleurothallis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU137-16	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU138-16	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU139-16 ^b	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU140-16	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU141-16	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU142-16	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU143-16	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU144-16	<i>Pleurothallis</i>	<i>variabilis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU145-16 ^a	<i>Ponthievia</i>	<i>sp.</i>	Orchidoideae	Cranichideae		Salazar et al., 2003
ECU146-16 ^b	<i>Prescottia</i>	<i>stachyodes</i>	Orchidoideae	Cranichideae		Salazar et al., 2003
ECU147-16 ^a	<i>Prescottia</i>	<i>stachyodes</i>	Orchidoideae	Cranichideae		Salazar et al., 2003
ECU148-16 ^b	<i>Prescottia</i>	<i>stachyodes</i>	Orchidoideae	Cranichideae		Salazar et al., 2003

ECU149-16 ^b	<i>Pseudocentrum</i>	<i>sylicola</i>	Orchidoideae	Cranichideae	Cranichidinae	Kolanowski and Szlachetko, 2015
ECU382-16 ^a	<i>Pelexia</i>	<i>sp.</i>	Orchidoideae	Cranichideae		Salazar et al., 2003
ECU151-16 ^b	<i>Sobralia</i>	<i>ecuadorana</i>	Epidendroideae	Sobralieae		Neubig et al., 2011
ECU156-16 ^b	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU157-16 ^b	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU158-16 ^b	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU159-16 ^b	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU160-16 ^b	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU161-16 ^b	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU152-16 ^b	<i>Stelis</i>	<i>piperina</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU153-16 ^b	<i>Stelis</i>	<i>piperina</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU154-16 ^a	<i>Stelis</i>	<i>pusilla</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU155-16 ^b	<i>Stelis</i>	<i>pusilla</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU162-16 ^b	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU163-16	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU164-16	<i>Stelis</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU165-16 ^a	<i>Teagueia</i>	<i>teaguei</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU166-16 ^{a,b}	<i>Telipogon</i>	<i>sarae</i>	Epidendroideae	Maxillareae	Oncidiinae	Williams et al., 2005
ECU167-16	<i>Telipogon</i>	<i>steinii</i>	Epidendroideae	Maxillareae	Oncidiinae	Williams et al., 2005
ECU169-16 ^b	<i>Trichosalpinx</i>	<i>sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU168-16 ^b	<i>Trichosalpinx</i>	<i>dirrhamphis</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU170-16 ^b	<i>Trichosalpinx</i>	<i>Sp.</i>	Epidendroideae	Epidendreae	Pleurothallidinae	Dressler (1993)
ECU171-16 ^b	unidentified	unidentified				
ECU172-16	unidentified	unidentified				
ECU173-16 ^b	unidentified	unidentified				
ECU174-16 ^b	unidentified	unidentified				
ECU175-16 ^b	unidentified	unidentified				
ECU176-16 ^b	<i>Xylobium</i>	<i>leontoglossum</i>	Epidendroideae	Maxillareae	Lycastinae	Dressler (1993)

^a Molecular samples taken from alcohol preserved herbarium specimens

^b Samples successfully sequenced for both matK and rbcL loci.

Table 3. Pairwise difference between samples in subtribe Pleurothallidinae.

CLADE B	CLADE D	Total number of differences
AP6992 Pleurothallis sclerophylla	KB169 Pleurothallis dunstervillei	14
CLADE B	CLADE B	Total number of differences
AP6992 Pleurothallis sclerophylla	KB122 Stelis piperina	4

We support the results of Kyle Dwyer as presented here.

Dec 5, 2016
Date

K. S. Burgess
Kerla S. Burgess, Associate
Professor of Biology, Thesis Advisor

12/5/2016
Date

Alvaro Ferra
Alvaro Ferra, Professor of Botany and
Curator of Angiosperms at Pontificia
Universidad Católica del Ecuador

5 Dec 2016
Date

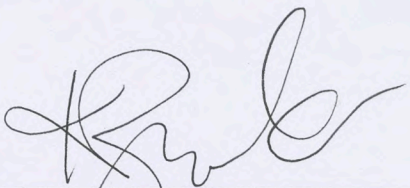
John L. Sanders
John L. Sanders, Vice President of
Science and Conservation,
Atlanta Botanical Garden

12/19/16
Date

Alex Reynolds
Alex Reynolds, Director of
Siempre Verde Reserve

I have submitted this thesis in partial fulfillment of the requirements for the degree of
Master of Science.

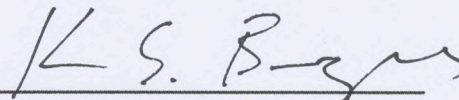
Dec 5, 2016
Date



Kylie Bucalo

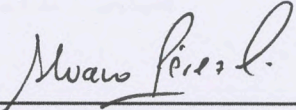
We approve the thesis of Kylie Bucalo as presented here.

Dec 5, 2016
Date



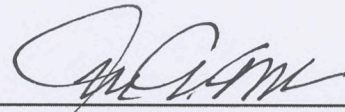
Kevin S. Burgess, Associate
Professor of Biology, Thesis Advisor

12/5/2016
Date




Alvaro Pérez, Professor of Botany and
Curator of Angiosperms at Pontificia
Universidad Católica del Ecuador

5 Dec 2016
Date



Jenny Cruse-Sanders, Vice President of
Science and Conservation,
Atlanta Botanical Garden

10/5/16
Date



Alex Reynolds, Director of
Siempre Verde Reserve

